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TRANSMITTAL LETTER

APPEAL BRIEF

Applicant	:	Moser, et al.
App. No	:	10/072,425
Filed	:	February 7, 2002
For	:	DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE
Examiner	:	Ewoldt, Gerald R.
Art Unit	:	1644

CERTIFICATE OF MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on

June 6, 2006
(Date)

Che S. Chereskin

Che Swyden Chereskin, Ph.D., Reg. No. 41,466

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith for filing in the above-identified application are the following enclosures:

(X) Appeal Brief in 31 pages with Attachments A through G.

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The present application qualifies for Small Entity Status under 37 CFR 1.27.

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Appeal Brief	*41.20(b)(2)*	2402 ($250)		$250
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02 FC:2251 60.00 OP

Docket No.	:	DECLE55.1C2CD1	**Customer No.: 20,995**
Application No.	:	10/072,425	
Filing Date	:	February 7, 2002	

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Dated: June 6, 2006

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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TC/A.U.	:	1644
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Docket No.	:	DECLE55.1C2CD1
Customer No.	:	20,995

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

APPEAL BRIEF

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

The Appellant appeals the rejection of Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-28, 30-35, 37-39 and 41-57 in the above-captioned application. These claims were rejected in a Final Office Action dated September 8, 2005.

This Appeal Brief is being filed in accordance with the rules of 37 C.F.R. § 41.37 and includes a Claims Appendix, an Evidence Appendix, and a Related Proceedings Appendix.

I. REAL PARTY IN INTEREST

The real parties in interest are Vrije Universiteit Brussel and Universite Libre De Bruxelles. The assignment of Kris Thielemans to Vrije Universiteit Brussel is recorded at Reel 012932/ Frame 0166. The assignment of the remaining inventors to Universite Libre De Bruxelles is recorded at Reel 012586/ Frame 0128.

II. RELATED APPEALS AND INTERFERENCES

A pre-Appeal Brief Request for Review and an Appeal Brief has been submitted for related Application No. 09/951,849.

III. STATUS OF CLAIMS

Claims 1-3, 5-14, 16-24, 26-35, 37-57 as listed the Claim Appendix, remain pending. Claims 3, 8, 19, 29, and 40 are withdrawn from consideration. Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-28, 30-35, 37-39, and 41-57 are the subject of this Appeal.

On September 8, 2005, the Examiner finally rejected Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-28, 30-35, 37-39, and 41-57.

Prosecution History Of Claims Prior To September 8, 2005 Final Office Action

The above-captioned application was originally filed on February 7, 2002, with Claims 1-57 as a divisional of U.S. Application No. 09/951,849, filed September 10, 2001, which is a continuation of 09/049,502, filed March 27, 1998, abandoned, which is a continuation-in-part of U.S. Application No. 09/025,405, filed February 18, 1998, abandoned, which is a continuation of Application No. 08/625,507, filed March 29, 1996, abandoned, which is a continuation-in-part of Application No. 08/414,480, filed March 31, 1995, abandoned.

On December 28, 2004, when responding to the election of species requirement of November 29, 2004, Appellants elected to prosecute claims pertaining to myeloid origin without amending the claims. However, in the Office Action of March 21, 2005 the Examiner indicated that upon reconsideration, DCs of lymphoid origin were rejoined.

On June 10, 2005, when responding to an Office Action mailed on March 21, 2005, Appellant amended Claims 1, 2, 10, 12, 21, 31, and 47-49. Claims 4, 15, 25, and 36 were cancelled. Claims 3, 8, 19, 29, and 40 remain pending but are withdrawn from consideration.

IV. STATUS OF AMENDMENTS

All amendments have been entered. No amendments have been submitted after the Final Office Action of September 8, 2005.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates to methods for producing a plurality of dendritic cell/tumor cell hybrids and methods of producing dendritic cell/tumor cell hybridomas by fusion of tumor cells with dendritic cells (DCs). DC/tumor cell hybrids and hybridomas produced according to the invention are useful in treatment of cancer.

In particular, claims 1-2, 5-7, 9, 42, 46, 50 and 54 are directed to fusion of tumor cells with autologous, HLA-compatible, or allogeneic dendritic cells to produce DC/tumor cell hybrids. These claims are supported generally at page 28, line 20-25 (Embodiments A & B).

Claims 10-14, 16-18, 20, 43, 47, 51, and 55 are directed to fusion of immortalized tumor cells with autologous, HLA-compatible, or allogeneic dendritic cells to produce DC/tumor cell hybridomas. These claims are supported generally at page 28, line 29 to page 29, line 5 (Embodiments F, G, H, and I).

Claims 21-24, 26-28, 30, 44, 48, 52, and 56 are directed to fusion of tumor cells with immortalized autologous, HLA-compatible or allogeneic dendritic cells to produce DC/tumor cell hybridomas. These claims are supported generally at page 28, line 29 to page 29, line 5 (Embodiments D & E).

Claims 31-35, 37-39, 41, 45, 49, 53, and 57 are directed to fusion of a tumor cell line that has at least one tumor-associated antigen in common with a tumor sample with autologous, HLA-compatible or allogeneic dendritic cells to produce DC/tumor cell hybridomas. These claims are supported generally at page 29, line 25 to page 30, line 13 (Embodiments J, K, L, and M).

Dendritic cells are defined on page 11, last paragraph. to page 12, first paragraph, as an isolated dendritic cell or its dendritic cell progenitor, preferably derived from bone marrow and obtained as described in Example 12. In said Example 12, DC fusion partners are prepared by differentiating in vitro proliferating DC precursors isolated from bone marrow.

As is well known, tumor cells generally do not act as antigen presenting cells (APCs) and do not elicit an immune response (page 5, paragraph 1). On the other hand, DCs "are considered as the natural adjuvant of the primary immune response...[and have an] unique ability to sensitize naive T-lymphocytes" (page 9, paragraph 2). The present invention is directed to DC/tumor cell hybrids which harness the ability of a DC to elicit an immune response to provide

an anti-tumor response (page 9, last paragraph). Tumor cells obtained from a cancer patient can be fused with a DC to produce a DC/tumor cell hybrid which is administered to the patient where the DC component of the hybrid facilitates an immune response against the tumor.

Example 12 (page 52, line 22 to page 66, line 19) teaches fusion of P815 tumor cells with bone marrow-derived dendritic cells. Out of 50 clones, one clone, hybrid 38, displayed morphological features of dendritic cells (page 58, 1st and 2nd full paragraphs) and also expressed characteristics of the P815 tumor cells such as expression of P1A (page 58, last paragraph to page 59 first paragraph). This clone represents a successful fusion between a dendritic cell and a tumor cell. The dendritic cell is derived from bone marrow and is not a T-lymphocyte or B-lymphocyte. The method used for Example 12 corresponds to the methods relating to independent claim 10.

Hybrid 38 (as well as bone marrow derived DCs) was successful in inducing a primary immune response including activation of naive T-cells As expected, the P815 tumor cells were not able to induce a primary response in vitro (page 59, first full paragraph).

Injections of the hybrid 38 cells into mice inoculated with a lethal dose of P815 tumor cells, protected the mice in 55% of the test animals (page 60, paragraph 2 and Figure 12), further confirming the immunological properties of the DC/ tumor cell hybrid identified as Hybrid 38. **A.**

Independent Claim 1

Claim 1. (Previously presented) A method for producing a plurality of dendritic cell/tumor cell hybrids which induce an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

(c) providing autologous or HLA-compatible allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood, and,

(d) fusing said dendritic cells with said tumor cells to produce a plurality of hybrids, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte.

B. Independent Claim 10

Claim 10. (Previously presented) A method for producing a dendritic cell/tumor cell hybridoma which induces an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary culture of said tumor sample to provide tumor cells,

(c) deriving an immortal cell line from said tumor cells to produce immortal tumor cells,

(d) providing autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(e) fusing said dendritic cells and said immortal tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(f) selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic cell morphology, dendritic-like cell or dendritic cell surface markers, dendritic cell genetic markers and immune cell activation in vitro.

C. Independent Claim 21

Claim 21. (Previously presented) A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response when applied to a patient causing the reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

(c) providing an immortal cell line comprising immortal autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in

vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(d) fusing said immortal dendritic cells with said tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(e) selecting from said plurality of hybridomas, a hybridoma which exhibits at least one characteristic selected from the group consisting of tumor cell morphology, tumor cell surface markers, and tumor cell chromosomal and genetic markers.

D. Independent Claim 31

Claim 31. (Previously presented) A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) analyzing tumor-associated antigens of said tumor sample,

(c) providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample,

(d) providing autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(e) fusing said dendritic cells with said immortal tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(f) selecting from said plurality of hybridomas, a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic cell morphology, dendritic cell surface markers, dendritic cell genetic markers and immune cell activation in vitro.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 1, 5, 6, 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28, and 29 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994, IDS) in view of Sornasse, et al. (1992).

2. Claims 2, 12, 33, 42, 43, 44, 46, 47, and 48 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994, IDS) in view of Sornasse, et al. (1992) as applied to Claims 1, 5, 6, 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28, and 29, and further in view of U.S. Patent No. 5,851,756.

3. Claims 50-52 and 54-56 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994, IDS) in view of Sornasse, et al. (1992) as applied to Claims 1, 4, 5, 6, 7, 9, 10, 11, 15, 16, 17, 18, 20, 21, 22, 25, 26, 27, 28, and 29, and further in view of U.S. Patent No. 5,637,483.

4. Claims 13, 14, 23, and 24 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994, IDS) in view of Sornasse, et al. (1992) as applied to Claims 1, 5, 6, 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28, and 29, and further in view of Reid, et al.

5. Claims 13, 14, 23, 24, 34, and 35 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

6. Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-28, 30-35, 37-39 and 41-57 are rejected under 35 U.S.C. § 112, first paragraph, as the specification does not contain a written description of the claimed invention in that the disclosure does not reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time the application was filed. This is a new matter rejection.

7. Claims 21-24, 26-31, 44, and 52 are rejected under 35 U.S.C. § 112, first paragraph as the specification does not contain a written description of the claimed invention, in that the disclosure does not reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time the application was filed. This is a new matter rejection.

VII. ARGUMENT

A. Rejections Under 35 U.S.C. § 103(a) Over Guo, et al. (1994, IDS) in View of Sornasse, et al. (1992).

The Examiner states that Guo, et al. (submitted here as Attachment A) teach production of hybrids/hybridomas by fusion of a bone marrow-derived antigen presenting B cell and a tumor cell. While Guo, et al. do not teach dendritic cells (DCs) as fusion partners, the Examiner asserts that it would be obvious to substitute a dendritic cell for the B-cell of Guo, et al. in view of Sornasse, et al. (submitted here as Attachment B).

1. B-cells are excluded as fusion partners.

The present claims on appeal have been amended to specifically exclude both B-lymphocytes and T-lymphocytes. Note that the present specification at page 11, line 24 suggests that B-cells are not to be used as fusion partners. At page 51, lines 21-24, the specification specifically states that a hybrid produced according to the claimed method did not include a B cell. The presently claimed invention is clearly non-obvious over Guo, et al. as the B-cells of Guo, et al. are specifically excluded. Sornasse, et al. do not provide sufficient motivation to substitute dendritic cells for the B-cells taught by Guo, et al. as discussed further below.

One of ordinary skill in the art at the time of the claimed invention would not be motivated to substitute a DC for the B-cell of Guo, et al. based upon the disclosure of Sornasse, et al. Sornasse, et al. conclude that in vitro antigen-pulsed DCs may be used as a physiological adjuvant in vivo (see Abstract). However, Appellants' method is directed to using DCs as fusion partners with tumor cells, not the use of DCs as a physiological adjuvant. Thus, there is no motivation to combine the references in the manner suggested by the Examiner.

The prior art must suggest the desirability of the claimed invention (see MPEP 2143.01, III).

The fact that the references can be combined or modified is insufficient to establish *prima facie* obviousness. The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990) (Claims were directed to an apparatus for producing an aerated cementitious composition by drawing air into the cementitious composition by driving the output pump at a capacity greater than the feed rate. The prior art reference taught that the feed means can be run at a variable speed, however the court found that this does not require that the output pump be run at the claimed speed so that air is

drawn into the mixing chamber and is entrained in the ingredients during operation. Although a prior art device "may be capable of being modified to run the way the apparatus is claimed, there must be a suggestion or motivation in the reference to do so." 916 F.2d at 682, 16 USPQ2d at 1432).

In the present case, Sornasse, et al teach the DCs "may" be used as a physiological adjuvant, but Sornasse, et al. is not using DCs as hybrids with other cells and does not suggest doing so. Guo, et al. only teach B-cell/tumor cell hybrids and does not suggest substitution of the B cells with any other cell type. Neither reference teaches the desirability of using DCs in DC/tumor cell hybrids. Accordingly, the combination of references does not provide any motivation to substitute B cells with DCs. Moreover, it is unpredictable that the desirable property which is the teaching of Sornasse, et al., this capability of initiating an immune response, would be retained after the DC is fused with a tumor cell. Neither reference addresses this point.

Even if there were a suggestion to combine the references, the substitution of DCs for B cells would, at most, be "obvious to try" in view of the teaching of Sornasse, et al. However, there would be no reasonable expectation of success because there is no teaching or suggestion in either reference that DCs could be isolated and combined with tumor cells to produce DC/tumor cell hybrids with anti-tumor activity.

As discussed in section 2a of the Moser I Declaration, previously submitted as Attachment B to the response filed June 10, 2005, for the present application (identical to Declaration I filed for the related application US 09/802,397; submitted here as Attachment C) it was not predictable at the time of the claimed invention that replacing the B-cells of Guo, et al. with DC cells would provide the DC/tumor cell hybrids of the claimed invention because it was known at the time of the claimed invention that fusion of dissimilar cells often resulted in loss of tissue specific traits.

Based upon the teaching of Sornasse, et al. one of ordinary skill in the art might expect DCs to function as a physiological adjuvant. However, Sornasse, et al. provide no basis to expect that DCs would retain their ability to induce an immune response after fusion with a tumor cell. Accordingly, one of ordinary skill in the art would not be motivated to substitute the B cells of Guo, et al. with DCs to obtain DC/tumor cell hybrids. There was no expectation,

based upon the cited references, that DC/tumor cell hybrids could be made and administered to produce an anti-tumor response.

The Examiner also asserts that the specification teaches that methods of fusing the DCs and tumors was an adaptation of a well-known method (Final Office Action, page 6, paragraph 2). While techniques to fuse cells were well known at the time of the claimed invention, DC/tumor cell hybrids capable of illiciting an immune response against the tumor were not known at the time of the claimed invention. It was not predictable at the time of the claimed invention that the fusion product would retain the ability of the DC to initiate an immune response against its hybrid partner which is a tumor cell. Yet this is what Applicants have achieved as exemplified in the present specification (see particularly page 60, line 1 to page 61 line 18 for hybrid 38).

2. The claims are limited to particular sources of DCs

The claims on appeal were amended to specify that the isolation of the DCs is "from bone marrow, lymph or blood", or from dendritic cells prepared "by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood".

The Examiner argues that "only a few of the dependent claims limit the source of the DCs to other than spleen" (pages 4-5 of the Final Office Action, bridging sentence). This is incorrect. All of the present claims are limited to "bone marrow, blood or lymph" (see claims 1(c), 10 (d), 21 (c), and 31 (d)).

Neither Guo, et al nor Sornasse, et al. teach isolation from bone marrow, blood or lymph. Neither Guo, et al. nor Sornasse, et al. teach DCs prepared "by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood" (claims 1, 10, 21, 31). Guo, et al. teach isolation of B-cells from spleen (see page 518, col. 1, last paragraph) and Sornasse, et al. teach isolation of DCs from spleen (see pages 15-16, bridging paragraph). However, the present application teaches that spleen is disfavored as a DC source for fusion with tumor cells. Although Examples 1-6 of the present application are directed to spleen as a source of DCs for DC/tumor cell hybrids, in fact, these experiments only produced a T-cell/tumor cell hybrid, which is outside the scope of the claims on appeal. As taught in the specification at page 66, lines 8-15, "[f]usion experiments have been performed using P815 and dendritic cells

isolated from spleen. The yield of hybrid clones was very low, as compared to fusions between P815 and bone-marrow derived DC, and none of them displayed phenotypic and functional features of dendritic cells, suggesting that fusion partners should be proliferating cells or dendritic cells at a more immature stage." Thus, reflecting the scope of the presently pending claims, the present application clearly teaches away from the use of spleen as a source for DCs for hybrids prepared according to the claimed method. Accordingly, even if one of ordinary skill in the art would have been motivated to substitute the B-cell of Guo, et al. with a DC (and Appellants maintain that the cited references do not provide such motivation), based upon both the disclosures of Guo, et al. and Sornasse, et al., one of ordinary skill in the art would have used spleen as the source of the DCs. As shown by the specification, DCs produced from spleen do not produce DC/tumor cell hybrids. Consequently, there was no reasonable expectation of success in achieving the invention as claimed by following the teachings of the two cited references.

The Examiner states that arguing that spleen is not a good source for DCs is a "particularly curious argument" given the fact that Examples 1-6 are directed to spleen and that this element is not recited in the claims (Final Office Action, page 5, 1st and 2nd full paragraphs). As discussed above, the claims do not include spleen as a source of DCs. While the first 6 examples were directed to spleen as a source for dendritic cells, these experiments were not successful and Appellants concluded that spleen is disfavored as a DC source in the practice of the claimed method. Although Examples 1-6 of the present application are directed to spleen as a source of DCs for DC/tumor cell hybrids, in fact, these experiments only produced a T-cell/tumor cell hybrid. As taught in the specification at page 66, lines 8-15, "Fusion experiments have been performed using P815 and dendritic cells isolated from spleen. The yield of hybrid clones was very low, as compared to fusions between P815 and bone-marrow derived DC, and none of them displayed phenotypic and functional features of dendritic cells, suggesting that fusion partners should be proliferating cells or dendritic cells at a more immature stage." Furthermore, all of the present claims are limited to "bone marrow, blood or lymph" (see claims 1(c), 10 (d), 21 (c), and 31 (d)). There is no recitation of DCs isolated from spleen in the claims on appeal.

As discussed in paragraph 9 of the attached Moser II Declaration, submitted as Attachment C to the Response filed June 10, 2005 (submitted here as Attachment E), spleen and lymph nodes contain a high proportion of differentiated DCs. Appellants discovered that these make poor fusion partners. This was not known by others at the time of the claimed invention but was discovered by the inventors of the present application and is clearly shown in the specification. Examples 1-6 of the present specification also demonstrate that DC/tumor cell hybrids could not be produced using spleen as the DC source.

Spleen cells either do not contain proliferating (differentiating) cells, or such cells are present in a negligible amount. This is consistent with the specification which shows that spleen cells are not a good choice for isolation of DC or DC precursors to make DC/tumor cell fusions. This was not known at the time of the invention which is why the initial experiments were performed (unsuccessfully) using spleen cells. The successful use of other sources such as bone marrow, lymph or blood is shown by the present specification and the Moser II Declaration (see paragraph 9) and is the focus of the claims on appeal.

The Examiner argues that the combined references, in view of what was known in the art at the time of the invention, render the methods obvious and that bone marrow, blood and lymph are the most well-known and obvious sources of DCs (Final Office Action, page 6, paragraph 2). The Examiner's assertion that the use of bone marrow, blood or lymph was an obvious choice for isolation of DCs is made without substantiation. Indeed, all of the references cited by the Examiner teach spleen as a source of DCs. The Examiner further argues that it would have been obvious to use DCs from bone marrow, blood or lymph because a patient would prefer to give blood rather than spleen for isolation of DCs to use according to the claimed method. However, as all of the art cited teaches the use of spleen as a source of DCs, one of ordinary skill in the art would not be motivated to use other sources. It is not the patient's motivation that is at issue, but rather the motivation of one of ordinary skill in the art to substitute DCs isolated from blood, bone marrow or lymph for the DCs isolated from spleen which are taught by Sornasse, et al. As discussed in section 1 above, such motivation is lacking in the combination of references cited by the Examiner.

The Examiner again points out that the specification teaches the use of DCs from spleen to form hybrids (Final Office Action, page 8, 2nd full paragraph). Again, while experiments with

DCs from spleen were carried out, the hybrids produced were not DC/tumor cell hybrids. The specification teaches away from using spleen as a source for DCs and spleen as a DC source is outside of the scope of the claims.

3. Routine experimentation would not lead to the present invention

In the Final Rejection of September 8, 2005, the Examiner argues that routine experimentation and variation in the method of Guo, et al. would be permissible and that "DC hybridomas retaining T cell activation capability were produced as early as 1981, (see, for example, the work of J.H. Peters)" (Final Office Action, page 4, lines 8-11). This statement is demonstrably false. DC hybridomas were **not** known before the filing of Appellants' application, and are certainly not shown by Peters. The Examiner has referred to no evidence other than Peters that DC hybridomas were known. While Peters has not been directly cited here, Peters has been cited in related application US 09/951,849. Peters does **not** teach DC/tumor cell hybrids for a number of reasons including the following:

a. Peters is not an enabling reference.

The reference cited in the related applications, Peters 1981 (Attachment F), is merely an Abstract and does not teach how the DCs were prepared. Peters 1981 refers to Peters 1980 (Attachment G) for details on the characteristics of the DCs used for the DC/tumor cell hybrids of Peters 1981. Both Peters 1981 and Peters 1980 cited therein are Abstracts only and do not contain sufficient detail for an enabling disclosure. The DC cells used in the method of Peters are only defined by their preparation procedure (line 5) and by some of their cellular characteristics (lines 6-7).

b. Peters does not teach cell hybrids prepared by fusing tumor cells to dendritic cells isolated from bone marrow, blood or lymph or to dendritic cells isolated by differentiating in vitro proliferating DC precursors isolated from bone marrow, blood or lymph.

The Peters reference does not describe preparation of the DC cells but refers to a previous publication (see above). This publication (submitted here as Attachment F) described the preparation of DC cells from spleen. The present claims specify that the dendritic cells are isolated from bone marrow, blood or lymph, not spleen. Peters does not teach isolation of dendritic cells from bone marrow, blood or lymph. Spleen cells contain differentiated DCs in

contrast to bone marrow, blood or lymph which contain proliferating DC precursors and DCs at a less mature stage as discussed above. Spleen cells are disfavored in the practice of the claimed invention (see page 66, lines 8-15 of the present specification)

c. Peters does not teach DC/tumor cell hybrids – Moser I Declaration

Dr. Moser is one of the inventors of the present application and is highly skilled in the immunology art as evidenced by the Moser I Declaration originally filed for the related application US09/951,849 (Submitted here as Attachment D). Based upon her review of the Peters 1981 Abstract cited by the Examiner and the Peters 1980 Abstract cited within the 1981 Abstract, Dr. Moser concludes that the hybridomas described by Peters do not have a dendritic cell component. As discussed in detail in the Moser I Declaration at section 2, Peters does not provide any evidence that the alleged DC/tumor hybridomas were made and does not teach how to make DC/tumor hybridomas.

For the reasons given above, Peters does not support the Examiner's assertion that DC/tumor cell hybrids that retain T cell activation characteristics were known at the time of the claimed invention.

4. The use of GM-CSF is nonobvious

The Examiner also argues that the use of GM-CSF in cell cultures is obvious. This refers to dependent claims 2, 12, 33, 42, 43, 44, 46, 47, and 48. As discussed below, Appellants maintain that the use of a cytokine such as GM-CSF is non-obvious because the Examiner has provided no teaching showing the use of a cytokine such as GM-CSF for differentiation of DCs in a method of making DC/tumor cell hybrids. U.S. Patent No. 5,851,756, which is cited below for this teaching, shows proliferation of DCs using GM-CSF, not differentiation.

5. The use of irradiation is nonobvious

The Examiner argues that the use of irradiation is obvious as it is unlikely that any patient would knowingly accept unattenuated or live tumor hybridomas. The Examiner is referring to dependent claims 50-52 and 54-56 which are discussed in a separate ground of rejection below. Appellants do not dispute that irradiation of tumor cells was known at the time of the claimed invention. Appellants maintain that fusion of DCs with tumor cells to produce DC/tumor cell hybrids and hybridomas was non-obvious at the time of filing of Appellants' claimed invention.

6. The present specification teaches DCs from bone marrow, blood and lymph and DCs differentiated in vitro from proliferating DC precursors isolated from bone marrow, blood or lymph.

The Examiner argues that the DCs used to produce clone 38 of Example 12 were not "the proliferating DCs of the claims" (Final Office Action, page 7, 3rd full paragraph). The DC fusion partner of clone 38 (Example 12) corresponds to DCs which were differentiated in vitro from bone marrow precursors through culturing DC precursors in vitro for 10 days (see present specification, page 53, last paragraph). A heterogenous cell population was obtained that included non-proliferating differentiated DCs and, probably to a lesser extent, proliferating DCs. Dendritic cells which are prepared by "differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood" are further supported by the specification (Embodiments B, G, I, K, and M on pages 28-30 and page 66, lines 13-15, for example) and enabled by Example 12 of the specification as discussed above and by the Moser II Declaration.

7. Hybrids prepared from differentiation of proliferating DC precursors are capable of illiciting an immune response

The Examiner argues that proliferating, less differentiated DCs of the claims would not be capable of producing the required response (Final Office Action, page 7, last paragraph). However, this ignores the evidence submitted with the Moser II Declaration.

The Moser II Declaration at paragraph 2 describes proliferation of bone marrow progenitors at different culture times by following various markers. Paragraph 4 describes fusion of early (3 and 4 day of culture) BMDC (bone marrow dendritic cells) compared to fully differentiated BMDCs (9 day culture). The Table in paragraph 5 confirms that the proliferating DC precursors (3 and 4 day of culture) are more efficient in DC/tumor cell fusions. The Moser II Declaration shows that less mature DCs are easier to fuse. Paragraph 6 shows that most of the clones that were fused to cells cultured for 3 days were true hybrids. Testing of 19 true hybrids showed that 13 exhibited strong immunostimulatory properties and 4 exhibited weak immunostimulatory properties (paragraph 8). The Moser II Declaration shows that the DC/tumor cell hybrids produced from DCs that were less mature (3 days in culture) were able to induce an immune response.

Accordingly, the Moser II Declaration provides evidence that claims directed to dendritic cells which are prepared by "differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood" are enabled. Appellants submit that the Moser II Declaration provides clear evidence that DC/tumor cell hybrids produced from proliferating DC precursors are capable of producing an immune response as shown particularly in paragraph 8 of the Moser II Declaration. This evidence directly rebuts the Examiner's assertion that immature DCs would not induce an immune response.

For the reasons given above, the present claims are believed to be non-obvious over Guo, et al. in view of Sornasse, et al.

B. Rejections Under 35 U.S.C. § 103(a) Over Guo, et al. (1994, IDS) in View of Sornasse, et al. (1992) and Further in View of U.S. Patent No. 5,851,756.

As explained in section 4 of the Moser I Declaration, filed for the present application on June 10, 2005 and for the related application US 09/802,397 on November 10, 2003, claims 2, 12, 33, 42, 43, 44, 46, 47, and 48 are patentable over U.S. Patent No. 5,851,756. The '756 patent teaches that GM-CSF promotes proliferation in vitro of precursor DCs (see col. 4, line 63 to col. 5, line 9; col. 13, line 66-67, for example), not differentiation as claimed.

Furthermore, since claims 2, 12, 33, 42, 43, 44, 46, 47, and 48 depend ultimately from claims 1, 10, 21, or 31, which are neither taught nor suggested by the cited references, the invention defined in claim 2, 12, 33, 42, 43, 44, 46, 47, and 48 is also patentably distinguished from the references, alone or in combination.

C. Rejections Under 35 U.S.C. § 103(a) Over Guo, et al. (1994, IDS) in View of Sornasse, et al. (1992) and Further in View of U.S. Patent No. 5,637,483.

U.S. Patent No. 5,637,483 was cited to show the use of irradiation of tumor cells in an anti-tumor vaccine to prevent proliferation of the tumor cells in the patient. Irradiation of tumor cells was known at the time of the claimed invention. However, since claims 50-52 and 54-56 depend from claim 1, 10, and 21, which are neither taught nor suggested by Guo et al in view of Sornasse, et al. as discussed above, the invention defined in claims 50-52 and 54-56 is also patentably distinguished from the references, alone or in combination.

D. Rejections Under 35 U.S.C. § 103(a) Over Guo, et al. (1994, IDS) in View of Sornasse, et al. (1992) and Further in View of Reid, et al.

Reid, et al. was cited to show the use of HAT medium for killing of unfused immortal tumor cells. This technique was known at the time of the claimed invention. However, since claims 13, 14, 23, and 24 depend from claim 10 and 21, which are neither taught nor suggested by Guo et al in view of Sornasse, et al. as discussed above, the invention defined in claims 13, 14, 23, and 24 is also patentably distinguished from the references, alone or in combination.

E. Rejections Under 35 U.S.C. § 112, Second Paragraph.

Claims 13, 14, 23, 24, 34 and 35 are rejected as lacking antecedent basis for "said drug." Appellants assert that the antecedent basis for "drug" is found within claims 13, 23 and 35 in the recitation of "drug-sensitive".

As set forth in MPEP 2173.05(e), the failure to provide explicit antecedent basis for terms does not always render a claim indefinite.

If the scope of a claim would be reasonably ascertainable by those skilled in the art, then the claim is not indefinite. *Ex parte Porter*, 25 USPQ2d 1144, 1145 (Bd. Pat. App. & Inter. 1992) ("controlled stream of fluid" provided reasonable antecedent basis for "the controlled fluid"). Inherent components of elements recited have antecedent basis in the recitation of the components themselves. For example, the limitation "the outer surface of said sphere" would not require an antecedent recitation that the sphere has an outer surface. >See *Bose Corp. v. JBL, Inc.*, 274 F.3d 1354, 1359, 61 USPQ2d 1216, 1218-19 (Fed. Cir 2001) (holding that recitation of "an ellipse" provided antecedent basis for "an ellipse having a major diameter" because "[t]here can be no dispute that mathematically an inherent characteristic of an ellipse is a major diameter").

In the present case, Appellants maintain that the present recitation is not indefinite as one could reasonably ascertain that the "drug" to which the immortal tumor cells are exposed is the same "drug" to which the tumor cells are "drug-sensitive." It is not necessary to change "drug-sensitive" to –sensitive to a drug—at its first occurrence in the claim to make the meaning clear to one skilled in the art.

F. Rejections Under 35 U.S.C. § 112, first paragraph – New Matter.

The Examiner states that the original specification and claims do not provide support for reducing the number of tumor cells in a patient in claims 1, 10 and 21 (A); fusion using PEG (claims 9, 20, 30 and 41) (B); and "providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample" in claim 31 (C).

Support for the reduction in the number of tumor cells in a patient (claims 1, 10, and 21) is found in the specification, for example at page 60, paragraphs 1 and 2 which reports that injections of the hybrid cells "prevented the growth of pre-established P815 mastocytoma and provided long term protection." When mice were inoculated with a lethal dose of P815 and subsequently received intraperitoneal injections of hybrid cells, long term tumor protection resulted in 55% of the animals (see Figure 12). In the untreated animals, the tumors grew and killed the animals. The treated mice were also protected against a second tumor challenge (page 60-61, bridging paragraph; Figure 13). More generic descriptive support is found on page 15, second full paragraph, of the present specification which discloses that "the term "activation of immune cells in vivo" refers to the immune rejection of a residual tumor, as measured by its reduction in size and by the survival of the patient, as shown for mice in Example 5C or Example 12.

Support for fusion using PEG (claims 9, 20, 30, and 41) is found throughout the Examples. See Example 3 (page 33, line 23), Example 9 (page 46, lines 21-25) and Example 12 (page 54, line 5). Appellants note that the use of PEG to promote cell fusion is well known and is widely used in the art. One of ordinary skill in the art would know that the use of PEG to promote cell fusion is widely applicable to virtually all cell types and not limited to the specific conditions of Examples 3, 9, and 12.

Support for providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample (claim 31) is found at page 25, lines 8-12, which teaches that "as an alternative, a pre-established immortal human tumor cell line can be used, provided that at least one of the tumor-associated antigens from the patients' tumor cells are matched to these pre-established immortal tumor cell." See also Embodiments J, K, L, and M at pages 29-30. A skilled person knows that, if no common antigen is present between the tumor cell line to produce the hybrid and the tumor cell present in the patient to be treated, the hybrids/hybridomas formed are of no value. Appellants submit that claim 31 is supported and enabled by the specification in light of what would be well within the skill level of one skilled in the art.

G. New Grounds of Rejections Under 35 U.S.C. § 112, first paragraph – New Matter (necessitated by Appellants' amendment).

The Final Office Action of September 8, 2005 asserts that Claims 21-24, 26-31, 44, and 52 are not supported for a method of producing DC/tumor cell hybrids which comprises providing an immortal cell line comprising immortal autologous or HLA compatible or allogeneic DCs by isolation of DCs from bone marrow, lymph or blood or preparing said DCs by differentiating in vitro proliferating DC precursors isolated from bone marrow, lymph or blood.

Appellants believe that this ground of rejection does not apply to claim 31.

In addition to the sections of the specification referred to at page 11, lines 11-16 of the June 10, 2005 response (page 25, lines 13-25, page 28, line 20 to page 30, line 13 and page 66, lines 13-14), Appellants direct attention to the specification at page 26, lines 1-18 which describes (2°) obtaining primary DC cells or DCs differentiated from blood, bone marrow or other tissues by culture in the presence of cytokines, (3°) deriving immortal DCs from primary cultured DCs, and (4°) deriving HAT-sensitive variants of these primary or immortal DCs lines to yield drug-sensitive immortal DCs. The fusion of such immortal DC cell lines is clearly supported on page 28, line 20 to page 30, line 13 as previously submitted.

H. Conclusion

In view of the foregoing arguments distinguishing Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-29, 33, 42-44, 46-48, 50-52 and 54-56 over the art of record, arguments directed to the definiteness of Claims 13, 14, 23, 24, 34 and 35, and arguments that claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-35, 37-39, and 41-57 are fully supported by the specification, Appellants respectfully request that the rejection of these claims be reversed.

Please charge any additional fees, including any fees for additional extensions of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

Dated: June 6, 2006

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CLAIMS APPENDIX

1. A method for producing a plurality of dendritic cell/tumor cell hybrids which induce an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

(c) providing autologous or HLA-compatible allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood, and,

(d) fusing said dendritic cells with said tumor cells to produce a plurality of hybrids, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte.

2. The method of claim 1 wherein the dendritic cells of step (c) are produced by culturing said precursors in the presence of cytokines.

3. The method of claim 1 wherein the dendritic cells of step (c) are members of an immortal cell line.

4. (Cancelled)

5. The method of claim 1 wherein the dendritic cell of step (c) is of myeloid origin.

6. The method of claim 1 wherein the dendritic cell of step (c) is of lymphoid origin.

7. The method of claim 1 wherein the dendritic cell of step (c) is an isolated dendritic cell.

8. The method of claim 1 wherein the dendritic cell of step (c) is a dendritic cell progenitor.

9. The method of claim 1 wherein the fusion of step (d) is carried out using PEG.

10. A method for producing a dendritic cell/tumor cell hybridoma which induces an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary culture of said tumor sample to provide tumor cells,

(c) deriving an immortal cell line from said tumor cells to produce immortal tumor cells,

(d) providing autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(e) fusing said dendritic cells and said immortal tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(f) selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic cell morphology, dendritic-like cell or dendritic cell surface markers, dendritic cell genetic markers and immune cell activation *in vitro*.

11. The method of claim 10 further comprising selecting from said plurality of hybridomas, a hybridoma which expresses at least one tumor-associated antigen in common between the immortal tumor cells and the tumor against which an immune response is needed.

12. The method of claim 10 wherein the dendritic cells of step (d) are produced by culturing said precursors in the presence of cytokines.

13. The method of claim 10 wherein the immortal tumor cells of step (c) are drug-sensitive, said method further comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

14. The method of claim 13 wherein said drug is hypoxyxanthine-aminopterin-thymidine (HAT).

15. (Cancelled)

16. The method of claim 10 wherein the dendritic cell of step (d) is of myeloid origin.

17. The method of claim 10 wherein the dendritic cell of step (d) is of lymphoid origin.

18. The method of claim 10 wherein the dendritic cell of step (d) is an isolated dendritic cell.

19. The method of claim 10 wherein the dendritic cell of step (d) is a dendritic cell progenitor.

20. The method of claim 10 wherein the fusion in step (e) is carried out using PEG.

21. A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response when applied to a patient causing the reduction of the number of tumor cells in said patient, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

(c) providing an immortal cell line comprising immortal autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(d) fusing said immortal dendritic cells with said tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(e) selecting from said plurality of hybridomas, a hybridoma which exhibits at least one characteristic selected from the group consisting of tumor cell morphology, tumor cell surface markers, and tumor cell chromosomal and genetic markers.

22. The method of claim 21 further comprising selecting from said plurality of hybridomas, a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic cell morphology, dendritic cell surface markers, dendritic cell genetic markers and immune cell activation in vitro.

23. The method of claim 21 wherein the dendritic cells of step (c) are drug sensitive, said method further comprising, after step (d), killing unfused drug-sensitive immortal dendritic cells by exposure to said drug.

24. The method according to claim 23 wherein said drug is hypoxanthine-aminopterin-thymidine (HAT).

25. (Cancelled)

26. The method of claim 21 wherein the dendritic cell of step (c) is of myeloid origin.

27. The method of claim 21 wherein the dendritic cell of step (c) is of lymphoid origin.

28. The method of clam 21 wherein the dendritic cell of step (c) is an isolated dendritic cell.

29. The method of claim 21 wherein the dendritic cell of step (c) is a dendritic cell progenitor.

30. The method of claim 21 wherein the fusion in step (d) is carried out using PEG.

31. A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising:

(a) providing a sample of a tumor against which said response is needed,

(b) analyzing tumor-associated antigens of said tumor sample,

(c) providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample,

(d) providing autologous or HLA-compatible or allogeneic dendritic cells by isolation of dendritic cells from bone marrow, lymph or blood, or, preparing said dendritic cells by differentiating in vitro proliferating dendritic cell precursors isolated from bone marrow, lymph or blood,

(e) fusing said dendritic cells with said immortal tumor cells to produce a plurality of hybridomas, wherein said dendritic cell is not a T-lymphocyte or B-lymphocyte, and

(f) selecting from said plurality of hybridomas, a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic cell morphology, dendritic cell surface markers, dendritic cell genetic markers and immune cell activation in vitro.

32. The method of claim 31 further comprising selecting from said plurality of hybridomas, a hybridoma which expresses at least one tumor-associated antigen in common between the immortal tumor cells and the tumor against which an immune response is needed.

33. The method of claim 31, wherein the dendritic cells of step (d) are produced by culturing in the presence of cytokines.

34. The method of claim 31, wherein said tumor cells of step (c) are drug sensitive, said method comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

35. The method according to claim 34 wherein said drug is hypoxanthine-aminopterin-thymidine (HAT).

36. (Cancelled)

37. The method of claim 31 wherein the dendritic cell of step (d) is of myeloid origin.

38. The method of claim 31 wherein the dendritic cell of step (d) is of lymphoid origin.

39. The method of claim 31 wherein the dendritic cell of step (d) is an isolated dendritic cell.

40. The method of claim 31 wherein the dendritic cell of step (d) is a dendritic cell progenitor.

41. The method of claim 31 wherein the fusion in step (e) is carried out using PEG.

42. A method of claim 1 wherein the obtained hybrid is further induced to express the dendritic cell characteristics.

43. A method of claim 10 wherein the obtained hybridoma is further induced to express the dendritic cell characteristics.

44. A method of claim 21 wherein the obtained hybridoma is further induced to express the dendritic cell characteristics.

45. A method of claim 31 wherein the obtained hybridoma is further induced to express the dendritic cell characteristics.

46. A method of claim 42 wherein said induction is performed using GM-CSF, IFN,γ, TNF-α or a combination thereof.

47. A method of claim 43 wherein said induction is performed using GM-CSF, IFN-γ, TNF-α or a combination thereof.

48. A method of claim 44 wherein said induction is performed using GM-CSF, IFN-γ, TNF-α or a combination thereof.

49. A method of claim 45 wherein said induction is performed using GM-CSF, IFN-γ, TNF-α or a combination thereof.

50. A method of claim 1 wherein the obtained hybrid is treated to prevent further proliferation before using it for the induction of an anti-tumor response.

51. A method of claim 10 wherein the obtained hybridoma is treated to prevent further proliferation before using it for the induction of an anti-tumor response.

52. A method of claim 21 wherein the obtained hybridoma is treated to prevent further proliferation before using it for the induction of an anti-tumor response.

53. A method of claim 31 wherein the obtained hybridoma is treated to prevent further proliferation before using it for the induction of an anti-tumor response.

54. A method of claim 50 wherein said treatment occurs by irradiation.

55. A method of claim 51 wherein said treatment occurs by irradiation.

56. A method of claim 52 wherein said treatment occurs by irradiation.

57. A method of claim 53 wherein said treatment occurs by irradiation

EVIDENCE APPENDIX

Attachment A: Guo, et al. (1994, IDS)

Attachment B: Sornasse, et al. (1992)

Attachment C: Moser I Declaration filed for the present application on June 10, 2005 as a response to the Office Action dated March 21, 2005 and for the related application US 09/802,397 on November 10, 2003.

Attachment D: Moser I Declaration for US 09/951,849. A similar, although not identical, Moser I Declaration was filed in related application US 09/951,849 on November 4, 2003.

Attachment E: Moser II Declaration filed for the present application on June 10, 2005 as a response to the Office Action dated March 21, 2005, and for the related applications US 09/802,397 and US 09/951,849 on February 4, 2005.

Attachment F: Peters 1981(Peters raised by Examiner in Final Office Action mailed September 8, 2005)

Attachment G: Peters 1980

RELATED PROCEEDINGS APPENDIX

Appeal Brief in related Application No. 09/951,849. An Appeal Brief was filed on April 28, 2006.

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ATTACHMENT A

ARG4 translation start), and the probe is Nco I–Bgl II (−4.3 kb to −3.4 kb relative to *ARG4*). *THR4* centromere-proximal (Fig. 2B): Bgl II (nts 204996 to 215453), and the probe is Hind III–Hind III (nts 214047 to 214935). The band in lanes 1 and 17 through 23 of Fig. 2B apparently located just after the start of *YCR84w* is artifactual and is due to contamination of the probe with an adjacent fragment. *YCL14w-NFS1* (Fig. 2C): *Xho*I (nts 90286 to 99224), and the probe is Kpn I–Eco RI (nts 91185 to 91571). *YCL4w-YCL11c* (Fig. 2D): Xho I (nts 99224 to 118089), and the probe is Eco RI–Hind III (nts 100317 to 103054).

30. Diploid strains homozygous for *pho80ΔX::LEU2* or *pho4Δ25::LEU2* (L. W. Bergman, personal communication) were constructed by transforming *leu2* derivatives of the haploid parents of NKY1002 with the appropriate deletion or disruption. Yeast DNA was extracted at the indicated time after induction of meiosis, digested with Eco RI (Eco RI cuts 2.6 kb upstream and >10 kb downstream of the *PHO5* translation start site), displayed on a 1.2% agarose gel, transferred to membranes, and hybridized with an Eco RI–Cla I fragment (−2.6 kb to −2.2 kb). DSB positions were determined with external standards (Bst EII digests of λ DNA) and internal standards comprising Eco RI/Sal I, Eco RI/Bst EII, and Eco RI and Bam HI double digests of DNA from mitotic cells (Sal I, Bst EII, and Bam HI cut at nts +74, −185, and −542, respectively). DSB frequencies were determined as described above.

31. We thank L. Bergman, G. Simchen, N. Sugawara, and L. Symington for plasmids; M. Mendel and C. Wu for advice; G. Simchen, M. Smith, and C. Szent-Györgyi for prompting this study; L. Bergman, N. Schultes, and R. Malone for permission to cite unpublished data; and D. Chattoraj, C. Klee, J. Haber, A. Segall, R. Simpson, C. Vinson, M. Yarmolinsky, and C. Wu for comments that improved the manuscript.

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Effective Tumor Vaccine Generated by Fusion of Hepatoma Cells with Activated B Cells

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Fusion of BERH-2 rat hepatocellular carcinoma cells with activated B cells produced hybrid cells that lost their tumorigenicity and became immunogenic. Syngeneic rats injected with BERH-2–B hybrid cells became resistant to challenge with parental BERH-2 cells, and rats with established BERH-2 hepatomas were cured by subsequent injection of BERH-2–B cells. Both $CD4^+$ and $CD8^+$ cells were essential for the induction of protective immunity; however, only $CD8^+$ cells were required for the eradication of BERH-2 tumors. The generation of hybrid tumor cells that elicit antitumor immune responses may be a useful strategy for cancer immunotherapy.

Tumor cells may escape immune surveillance because they do not express signals that are essential for activation of the host immune system (*1*, *2*). At the molecular level, the defective signaling of tumor cells could be attributable to (i) down-regulation of major histocompatibility complex (MHC) molecules (*3*, *4*); (ii) alteration of antigen-processing pathways, resulting in an inability to present tumor-specific antigens to host T cells (*5*); (iii) absence of costimulatory or adhesion molecules that are essential for activation of the host immune system (*6*); or (iv) production of factors that modify host immune responses (*7*).

Activated B cells are the most effective antigen-presenting cells (*8*). We hypothesized that fusion of a tumor cell with an activated B cell would produce a hybridoma that both expressed tumor-specific antigens and had the machinery for antigen presentation and T cell activation.

BERH-2 is a chemical carcinogen–induced hepatocarcinoma from the Wistar rat (*9*). Cells derived from this tumor grow rapidly and form tumors in the liver of syngeneic animals. We obtained activated B cells from the spleens of rats injected 14 days earlier with bovine serum albumin in Freund's complete adjuvant (*10*). BERH-2 cells were fused with purified activated B cells by treatment with polyethylene glycol (PEG) (*11*). The fused cells were enriched by selection with a rabbit antiserum to BERH-2 cells and subsequent selection with a rabbit antiserum to rat B cells (*10*).

The parental BERH-2 cells expressed low amounts of MHC class I antigens and intracellular adhesion molecule–1 (ICAM-1) but were devoid of MHC class II antigens, leukocyte functional antigen–1 (LFA-1), and costimulatory molecule B7. In contrast, the BERH-2–B hybrid cell lines expressed MHC class II antigens, ICAM-1, LFA-1, and B7 (Fig. 1) and had enhanced expression of MHC class I antigens. These BERH-2–B cell lines have stably expressed both tumor and B cell antigens for more than 10 months.

All 10 rats injected intrahepatically with 2×10^6 parental BERH-2 cells developed liver tumors and died within 60 days (*12*). In contrast, 10 rats injected with the same number of BERH-2–B hybrid cells remained tumor-free for 180 days (*12*). All four hybrid

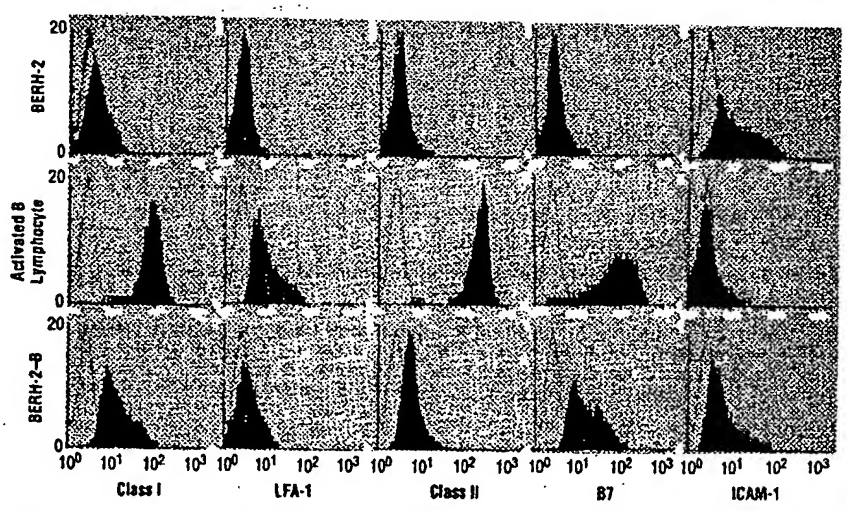

Fig. 1. Expression of MHC class I and class II antigens, B7, ICAM-1, and LFA-1 on BERH-2 cells, activated B cells, and BERH-2–B hybrid cells. Cells were washed with phosphate-buffered saline (PBS) and stained with monoclonal antibodies to rat MHC class I (OX-18), MHC class II (OX-6), ICAM-1 (IA 29), or LFA-1 (WT.1). To stain for rat B7, we used CTLA4-Ig, a soluble fusion protein containing the variable domain of the human CTLA-4 protein and the hinge, CH2, and CH3 domains of the human IgG1 constant region (*14*). Cells were incubated with the antibodies or chimeric protein for 30 min on ice. A mouse antibody to human CD3 (GH3, IgG2b) and a soluble human CD44-Ig chimeric protein were used as negative controls. Cells were washed three times. Fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse Ig or FITC-labeled rabbit antibody to human Ig was added for another 30 min on ice. Samples were then washed, fixed, and analyzed in a FACScan (Becton Dickinson, San Jose, California). Solid areas are cells stained with specific antibodies. Open areas are cells stained with control antibodies.

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cell lines we have examined lost their ability to form tumors in syngeneic rats. However, the hybrid tumor cells were able to grow and form tumors in nude mice (*12*).

Rats injected with hybrid BERH-2–B cells developed abundant lymphocytic infiltrates around the injected tumor cells (*12*). Most of the infiltrating cells were T lymphocytes. About 70% of these cells were $CD8^+$ and about 30% were $CD4^+$, as revealed by immunofluorescence staining of tissue sections with monoclonal antibodies specific for rat CD8 or CD4. There was no inflammatory response in animals injected with parental BERH-2 cells (*12*).

The rats that were immunized with BERH-2–B hybrid cells did not support growth of parental BERH-2 cells. All animals immunized with the BERH-2–B cells remained tumor-free for more than 150 days after challenge with BERH-2 cells. However, all rats immunized subcutaneously with BERH-2 cells or irradiated BERH-2 cells and then challenged intrahepatically with BERH-2 cells developed tumors and died within 60 days (Fig. 2A). Immunization with BERH-2–B cells could also eradicate established hepatomas. The rats that were injected with parental BERH-2 cells and then treated with BERH-2–B cells survived for more than 120 days. In contrast, rats injected and treated with the parental BERH-2 cells all died within 42 days (Fig. 2B). In other experiments, we surgically implanted small fragments of a BERH-2 hepatoma into the liver of rats (9). After 10 days, a fraction of the tumor-implanted animals were injected with BERH-2–B cells and the others were injected with parental BERH-2 cells. All rats injected with BERH-2 cells died within 50 days. Two of the eight rats injected with BERH-2–B hybrid cells developed tumors and died at 71 and 74 days after tumor implantation. The other six animals lived for more than 180 days after tumor implantation (Fig. 2C).

We next determined whether tumor rejection induced by BERH-2–B cells was mediated by $CD4^+$ or $CD8^+$ T cells. Rats were depleted of $CD4^+$ or $CD8^+$ cells by antibody treatment before injection of BERH-2–B cells. BERH-2–B cells were able to form tumors in CD4- and CD8-depleted rats (Table 1). In another experiment, rats were immunized with BERH-2–B cells, depleted of $CD4^+$ or $CD8^+$ cells 14 days later, and then challenged with BERH-2 cells. Tumors developed in rats depleted of $CD8^+$ cells but not in those depleted of $CD4^+$ cells (Table 1). Thus, both $CD4^+$ and $CD8^+$ cells appear to be necessary for the induction of immunity, but after the induction of the immune response, $CD8^+$ cells can mediate tumor cell destruction in the absence of $CD4^+$ cells. These results differ from those obtained with murine melanomas, where it has been shown that tumor cells transfected with the B7 gene can directly stimulate naive $CD8^+$ T cells without the participation of $CD4^+$ T cells (6).

To investigate whether the immunity induced by BERH-2–B cells was tumor-specific, we tested the effect of these cells on NBT-II,

Table 1. Effects of depletion of $CD4^+$ or $CD8^+$ T cells on the growth of BERH-2–B and BERH-2 cells in vivo.

Antibody specificity	Treatment protocol		Number of animals with tumors
	Ab, then immunize*	Immunize, then Ab†	
None	–		0/6
CD4	+		4/6
CD8	+		5/6
Control Ab	+		0/6
CD4		+	0/5
CD8		+	5/5
Control Ab		+	0/5

*Female Wistar rats were treated with purified mouse monoclonal antibodies (Abs) to rat CD4 (OX-38) or CD8 (OX-8) or a control mouse monoclonal antibody to diethylthiamine pentaacetic acid. Each animal received 500 μg of the purified antibody intravenously twice per week for 3 weeks. Two days before injection of tumor cells, peripheral blood lymphocytes were obtained from individual, treated rats and stained with antibodies to CD4 or CD8 to verify the depletion of $CD4^+$ or $CD8^+$ T cells. Treatment with the antibody to CD4 depleted >95% of the $CD4^+$ cells, treatment with the antibody to CD8 depleted ~95% of the $CD8^+$ cells, and treatment with the control antibody did not alter the number of $CD4^+$ or $CD8^+$ cells. Three days after the last injection of antibodies, all rats were injected intrahepatically with 5×10^6 BERH-2–B tumor cells. †Female Wistar rats were immunized with 2×10^6 BERH-2–B cells injected subcutaneously. Two weeks after immunization, animals were treated with an antibody to CD4, an antibody to CD8, or a control antibody. The depletion of each cell type was verified by immunofluorescence staining. Three days after the last injection of the antibodies, all animals received 5×10^6 BERH-2 cells intrahepatically. These experiments were repeated twice with comparable results.

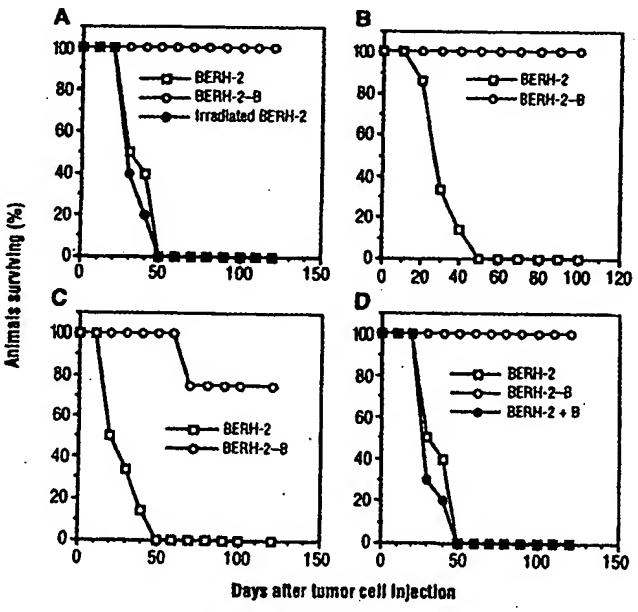

Fig. 2. Induction of protective immunity with BERH-2–B hybrid tumor cells or nonselected BERH-2–B hybrid tumor cells. (**A**) Three groups of female Wistar rats (eight per group) were injected subcutaneously with 2×10^6 BERH-2 cells, BERH-2–B cells, or irradiated (5000 roentgens) BERH-2 cells. After 2 weeks, all three groups were challenged with 5×10^6 BERH-2 cells injected intrahepatically. This experiment was repeated twice with identical results. (**B**) Fourteen rats were injected intrahepatically with 2×10^7 BERH-2 cells. Ten days later, eight of the injected rats were immunized with a subcutaneous injection of 5×10^6 BERH-2–B hybrid cells. The other six rats were injected subcutaneously with the same number of BERH-2 cells. This experiment was repeated twice with identical results. (**C**) Fourteen rats were intrahepatically implanted with a small fragment (0.3 mm by 0.5 mm) of BERH-2 tumor. After 10 days, eight of the animals were injected subcutaneously with 5×10^6 BERH-2–B hybrid cells. The other six rats were injected subcutaneously with the same number of BERH-2 cells. This experiment was repeated twice with identical results. (**D**) Three groups of rats (eight per group) were injected subcutaneously with 5×10^6 BERH-2 cells, 5×10^6 BERH-2 cells fused with 5×10^6 activated B cells, or 5×10^6 BERH-2 cells mixed with 5×10^6 activated B cells in the presence of PEG. Fused cells were washed three times with PBS, resuspended in PBS, and injected subcutaneously. Two weeks later, all rats were challenged with 5×10^6 BERH-2 tumor cells intrahepatically. This experiment was repeated three times with comparable results.

Table 2. Specificity of the immune response elicited by BERH-2–B hybrid tumor cells. Female Wistar rats were injected subcutaneously with 2×10^6 BERH-2–B cells. Two weeks after immunization, one group of rats was injected intrahepatically with 5×10^6 BERH-2 cells. Another group of rats was injected subcutaneously with 5×10^6 NBT-II rat bladder carcinoma cells (obtained from American Type Culture Collection). Tumors developed locally in the subcutaneously injected site in all rats immunized with BERH-2–B tumor cells and challenged with NBT-II tumor cells. All animals in this group died within 45 days after tumor cell challenge. This experiment was repeated twice with identical results.

Immunization cells	Challenge cells	Number of animals with tumors
BERH-2–B	BERH-2	0/8
BERH-2–B	NBT-II	8/8

a bladder carcinoma that grows rapidly in syngeneic Wistar rats. Immunization with BERH-2–B cells did not inhibit the growth of NBT-II cells in vivo (Table 2). In addition, $CD8^+$ T cells from rats immunized with BERH-2–B lysed BERH-2 cells but not NBT-II cells in vitro (*12*).

Finally, we determined whether in vitro selection of hybrid tumor cells was obligatory for the induction of tumor immunity. After fusing BERH-2 tumor cells with activated B cells, we washed the mixture of cells and injected them subcutaneously into syngeneic rats without prior in vitro selection. The efficiency of the fusion ranged from 30 to 50%. For controls, we injected BERH-2 tumor cells mixed with activated B cells in the absence of PEG. All animals were then injected with the parental BERH-2 cells intrahepatically. Only animals immunized with tumor cells fused with activated B cells were protected from tumor formation. Simply mixing tumor cells with activated B cells was not effective in inducing protective immunity (Fig. 2D), nor was treating BERH-2 tumor cells with PEG alone (*12*).

In summary, a BERH hepatocarcinoma–specific vaccine in rats can be made by fusing tumor cells with syngeneic, activated B cells. In addition to MHC class II and B7 antigens, BERH-2–B cells may express other cell surface molecules that are essential for the stimulation of host T cells. Production of B cell–specific cytokines by hybrid tumor cells may be important in the elicitation of host immune responses (*13*). BERH-2 cells fused with activated T cells were unable to stimulate BERH-2–specific immune responses (*12*). Preliminary experiments suggest that tumor cells fused with activated allogeneic B cells are also immunogenic and can induce protective immunity (*12*).

In order to induce protective immunity, the hybrid tumor cells must retain their capacity to express tumor-specific antigens. In addition, the hybrid tumor cells must be able to process and present tumor-specific antigens so as to activate host T cells. Whether this approach can be used in other tumor models remains to be determined. Our observation that protective immunity can be induced by tumor cells fused with activated B cells without in vitro selection may have broad clinical applications and may provide a useful strategy for cancer immunotherapy.

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Parallel Neuronal Mechanisms for Short-Term Memory

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Although objects that have just been seen may persist in memory automatically for a time and interact passively with incoming stimulation, some tasks require that the memory be actively maintained and used. To test for the existence of separate automatic and volitional mechanisms of short-term memory, recordings were made from neurons in the inferior temporal cortex of monkeys while the monkeys held a sample picture "in mind" and signaled when it was repeated in a sequence of pictures, ignoring other stimulus repetitions. Some neurons were suppressed by any picture repetition, regardless of relevance, whereas others were enhanced, but only when a picture matched the sample. Short-term memory appears to reflect the parallel operation of these two mechanisms—one being automatic and the other active.

Combined evidence from psychology and neuroscience has cleaved long-term memory into two functionally independent systems: an explicit system for facts and events, and an implicit system for the learning of perceptual and motor skills and habits (*1*). Psychological studies suggest that there may be more than one neural system mediating short-term memory (STM) as well. Some theoretical accounts, for example, posit that incoming stimuli are automatically held in some type of short-term storage buffer but may, in addition, be voluntarily maintained by active rehearsal mechanisms (*2*). We sought neurophysiological evidence for multiple STM mechanisms in recordings from the anteroventral portion of the inferior temporal (IT) cortex, a region important for visual memory in primates, including humans (*3*).

Nearly all behavioral and physiological studies of memory in the IT cortex have used some variation of the delayed matching-to-sample (DMS) task, in which the subject indicates whether a test stimulus matches a previously shown sample stimulus. The memory of the sample has a lasting effect on many IT neurons, because their response to subsequent test items is suppressed according to how well they match the sample—a property we have termed "adaptive mnemonic filtering" (*4*, *5*). Because the sample is behaviorally relevant in DMS tasks, it is commonly assumed that it is actively maintained in memory (that is, "working memory"), interacting with the neural processing of incoming test stimuli; however, it is also possible that all stimuli, relevant or not (including, but not limited to, the sample), automatically linger in memory for a time, interacting with incoming stimuli. For example, if one actively searches for a repetition of the sample number 3897 in the following series—1436 3482 3482 3897—one may automatically detect the repeated but irrelevant number 3482, in addition to detecting the specific repetition of the sample number. Thus, detection of stimulus repetition in DMS tasks might be mediated by either automatic or active mnemonic mechanisms, or both.

To distinguish among these possibilities, we tested two monkeys with two types of trials (Fig. 1). The first type, standard trials, were conventional DMS trials identical to those used in our previous studies of adaptive

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ATTACHMENT B

Antigen-pulsed Dendritic Cells Can Efficiently Induce an Antibody Response In Vivo

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Summary

The aim of this study was to develop an immunization procedure avoiding external adjuvant. Data are presented showing that syngeneic dendritic cells (DC), which have been pulsed in vitro with antigen, induce a strong antibody response in mice. By contrast, antigen (Ag)-pulsed low-density B cells, although equally able to induce interleukin 2 secretion by an Ag-specific T cell hybridoma in vitro, only weakly prime the mice in vivo. Moreover, we show that the injection of Ag-pulsed DC induces the synthesis of isotypes similar to the immunoglobulin classes detected after immunization with the same Ag in complete Freund's adjuvant. Importantly, high amounts of IgG2a antibodies are produced, suggesting that T helper type 1 cells are activated. Collectively, these data indicate that DC can initiate a primary humoral response and that they may be used as physiological adjuvant in vivo.

The induction of immune responses in vivo is typically performed with antigens administered in artificial adjuvants, like alum and CFA (1). However, the discovery of dendritic cells (DC)[1] (2), which most efficiently activate a primary T cell response in vitro (3), suggests that these cells could be used to induce antibody responses in situ, avoiding the use of toxic adjuvants. These cells have some unique properties in vivo, as compared with the other APC: they are widely distributed in lymphoid as well as in nonlymphoid tissues (reviewed in reference 4); they seem to be the major source of processed antigen in vivo (5); and they home to the T-dependent region of lymph nodes and spleen (6, 7).

DC appear to play a major role in initiating various T cell immune responses in vivo, such as contact sensitivity (8–10), allograft rejection (11–14), and activation of MHC-restricted T cell responses (15). Taken together, these results suggest that injection of in vitro pulsed DC may provide an efficient way for inducing T-dependent immunity without the use of external adjuvant.

Little is known, however, about the induction by DC of specific B cell responses in vivo. Indeed, although it has been shown in our laboratory that mouse dendritic cells, pulsed in vitro with tobacco mosaic virus, could strongly enhance the primary and the secondary responses to the virus (16), similar data could not be obtained with other antigens like soluble proteins.

Recent studies (15, 17) clearly showed that the capacity of the DC population to process and present proteins was downregulated when the dendritic cells matured in culture. In this report, we show that injection of DC appropriately pulsed in vitro with soluble protein antigens induces strong specific humoral responses in vivo.

Materials and Methods

Mice. Female DBA/2 mice (H-2^d), 6–8 wk old, were purchased from Charles River Wiga (Sulzfeld, Germany).

Antigens. The antigens used in this study were Myoglobin from sperm whale skeletal muscle and gamma-globulin from human blood, fraction II (both from Fluka Chemie AG, Buchs, Switzerland).

Culture Medium. The complete culture medium used in all experiments was RPMI 1640 (Seromed; Biochem KG, Berlin Germany) supplemented with 10% FCS (Byosis S.A., Compiègne, France), penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME and L-glutamine (Flow ICN Biomedicals Bucks, UK).

T Cell Hybridoma. The I-E^d-restricted, myoglobin-specific hybridoma 13-26-8-HG.1 was derived by Dr. A. Livingstone (Basel Institute of Immunology, Basel, Switzerland).

Antigen-presenting Cells. Spleens were digested with collagenase (CLS III; Worthington Biochemical Corp., Freehold, NJ) and sepa-

[1] *Abbreviations used in this paper:* Ag, antigen; DC, dendritic cell; HGG, human gamma globulin.

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rated into low and high density fractions on BSA gradient (Bovuminar Cohn fraction V powder; Armour Pharmaceutical Co., Tarrytown, NY). The spleen DC were purified according to a procedure described by Crowley et al. (18). Briefly, low-density cells were cultured during 2 h in 10% FCS-containing medium and the nonadherent cells were removed by vigorous pipetting. The same procedure was repeated with a shorter (1 h) incubation without FCS. After overnight culture, nonadherent cells contain at least 90% of dendritic cells (as assessed by morphology and specific staining). To obtain low-density B cells, the nonadherent cells obtained after the 2-h incubation (before vigorous pipetting) were depleted of T cells. For convenience, these low density, nonadherent, T-depleted spleen cells will be called low-density B cells.

Antigen Pulsing of APC. For dendritic cell pulsing, the adherent cells of the low-density fraction of spleen were cultured overnight in complete medium containing 100 μg/ml of antigen. The nonadherent cells were then collected and were mainly DC (referred to as antigen [Ag]-pulsed DC). The low-density B cells were cultured in the same conditions and all cells were collected after overnight culture.

IL-2 Production by T Cell Hybridoma. The T cell hybridoma was cultured with varying numbers of APC in the presence of intact protein in complete culture medium in 96-well flat-bottomed microtiter plates. After a 24-h culture period, the supernatants were assayed for IL-2 using an IL-2-sensitive subline of the CTLL cell line (19). A total of 10^4 cells were incubated with the supernatants and 0.5 μCi [^{3}H]TdR was added per well during the final 6–16 h of culture. Cells were harvested, and incorporation of radioactivity was assessed as described above.

Determination of Antigen-specific Antibody Levels. Serum levels of antigen-specific antibodies were determined by ELISA according to standard procedures using polyclonal goat anti-mouse IgG reagent (Boehringer Mannheim Biochemicals, Mannheim, Germany) or isotype-specific rat mAbs (20). Antibody titers were calculated based on linear regression analysis of the optical densities. Results are expressed as titers determined using the midpoint of the titration curves relative to an internal standard run in each assay.

Immunization Protocols. For primary response, mice received an intravenous injection of 3 × 10^5 Ag-pulsed syngeneic DC. Control mice were injected with unpulsed DC, Ag-pulsed low-density B cells, or were left untreated. 5 d later, all mice received a boost of 100 μg of soluble antigen intravenously, except one group of untreated mice which was injected intraperitoneally with 100 μg of antigen emulsified in CFA. All mice were bled 7 or 8 d after the antigen boost. For secondary response, all groups of mice received an injection of 100 μg of soluble antigen intravenously 1 mo after the initial treatment and were bled 7 d later.

Results

Fresh Splenic Dendritic Cells Present Native Proteins In Vitro. The initiation of any T cell response requires two independent steps: Ag presentation and T cell sensitization. Ag presentation generates the ligand that is recognized by the α/β heterodimer of the clonally specific portion of the TCR for Ag, and usually requires the generation of small peptides. Although it is clear that purified splenic DC have a poor, if any, capacity for processing, data from Romani et al. (17) clearly showed that handling of intact proteins was down-regulated in cultured as compared with fresh epidermal Langerhans cells. More recently, Inaba et al. (15) showed that fresh splenic DC were able to process native proteins early, i.e., during the purification procedure of the DC. In a preliminary experiment, we compared the ability of dendritic cells and control cells to process and present myoglobin in vitro. We used a T cell hybridoma, since its activation only requires TCR occupancy, i.e., the presence of the appropriate antigen in the context of self MHC, and does not depend on any costimulatory signal (21). As control APC, we chose cells that were isolated from the same low-density fraction as the dendritic cells, but were nonadherent during the 2-h culture and were depleted of T cells (see Material and Methods). Fig. 1 shows that both APC populations (DC or low-density B cells), pulsed during overnight culture, strongly induce IL-2 secretion by a myoglobin-specific T cell hybridoma. The two types of APC, however, have distinct properties. Indeed, 24-h-old, purified DC cultured with antigen only slightly induce the activation of the T cell hybridoma, whereas 24-h-old low-density B cells very efficiently present the antigen in the same conditions.

Dendritic Cells Pulsed In Vitro with Antigen Induce a Specific Humoral Response In Vivo. To evaluate the ability of DC to induce an in vivo humoral response, syngeneic DC were pulsed with myoglobin during overnight culture and 3 × 10^5 cells

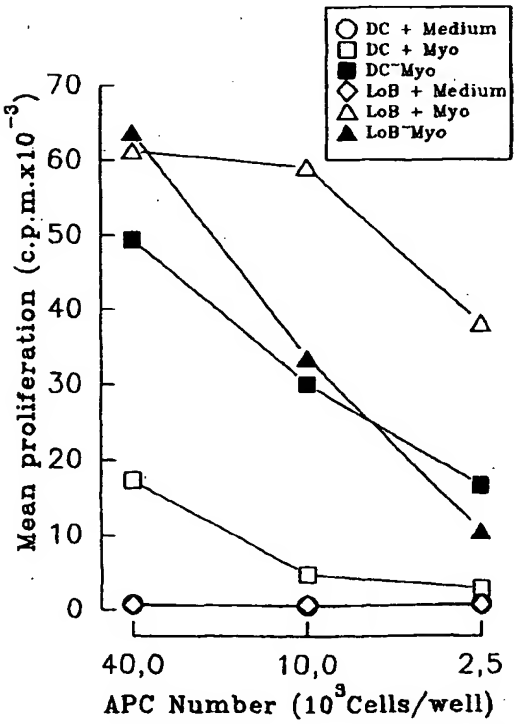
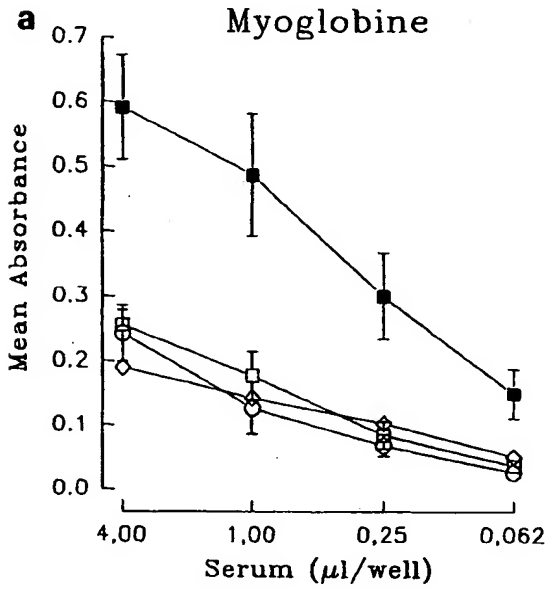


Figure 1. Fresh DC process native antigen in culture. Fresh DC and low-density B cells were cultured overnight in complete medium containing myoglobin (*DC∿myo* and *LoB∿myo*). Cells were washed, counted, and cultured with 3 × 10^4 T-T hybridoma cells (13-26-8). 24-h culture supernatants were assayed for IL-2 production in response to antigen-specific stimulation. Control APC (*DC* and *LoB*) were cultured without Ag during the purification steps and were cultured with hybridoma cells with (*+ Myo*) or without (*+ medium*) addition of antigen. Three experiments were performed with similar results.

were injected intravenously into syngeneic DBA/2 mice. 5 d later, animals were injected with 100 μg of antigen in saline. Control groups included mice injected with soluble antigen only, and mice that received unpulsed DC and the antigen



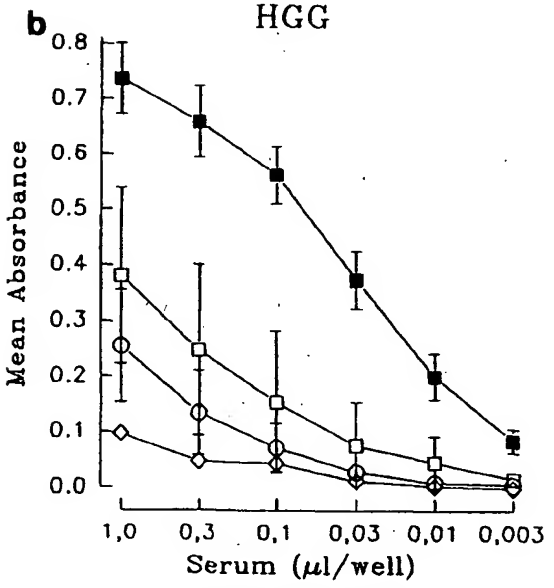


Figure 2. Antigen-pulsed DC induce a primary humoral response in vivo. DBA/2 mice (at least five per group) were injected with 3×10^5 myoglobin-pulsed (*a*) or HGG-pulsed (*b*) DC and boosted with 100 μg of the same antigen in saline (*closed squares*). Control groups included untreated mice (*diamonds*), mice injected with unpulsed DC (*open squares*), and mice that only received soluble Ag (*circles*). All mice were bled 8 d after antigen boost, and antigen-specific antibodies were measured in individual sera using a goat anti-mouse Ig reagent.

boost. The data in Fig. 2 *a* show that in vivo administration of Ag-pulsed DC induced a strong humoral response upon challenge with soluble antigen, whereas control mice produced little specific antibodies. Similar data were obtained with human gamma globulin (HGG): DBA/2 mice injected with HGG-pulsed DC and boosted with the same antigen in saline synthesized high amounts of antibodies specific for HGG (Fig. 2 *b*).

The Isotype Pattern Reflects the Activation of Th1 Cells In Vivo Recent studies indicate that unique cofactors are necessary for the selective activation of cloned murine $CD4^+$ cells representing Th1 and Th2 cells and that these factors are produced by specialized APC populations (22). Since the regulation of isotype switching in vivo is dependent upon the activation of different types of T helper cells (23), we investigated the class distribution of the specific antibodies. Fig. 3 summarizes the isotypes of the myoglobin-specific antibodies from mice tested individually in two independent experiments. The data show that high concentrations of IgG1 and variable but elevated amounts of IgG2a are secreted in the primary and secondary responses of DBA/2 mice primed with antigen-pulsed syngeneic DC. The injection of Ag-pulsed low-density B cells induces a low primary response, but the level of specific antibodies is increased after the antigen boost. Similar data were obtained in three independent experiments for the primary and secondary responses specific for HGG: Fig. 4 shows that HGG-pulsed DC are as potent as CFA in inducing specific antibody responses of IgG1 and IgG2a isotypes.

Discussion

The major observation from this study is that syngeneic DC that have been pulsed in vitro with native proteins induce a strong specific B cell response in vivo in unprimed animals that are boosted with soluble antigen. Thus, extracorporeal pulsing of DC may provide a physiological pathway for inducing T-dependent humoral responses in vivo.

The priming of naive animals by using elements of the immune system itself offers several advantages. It avoids the toxicity and the nonspecific immune activation often associated with the use of artificial adjuvants. Moreover, DC appear to very efficiently generate the antigenic epitopes (24, 25) that can be presented by self-MHC, and finally, the injection of DC promotes a specific memory B cell response.

The potent "adjuvant" capacity of DC, as compared with low-density B cells, correlates with their unique properties in vivo (for review, see reference 4). In particular, by down-regulating antigen processing (15, 17; Fig. 1), DC may not displace the acquired antigen with other antigens or with self proteins. This property appears to confer on the DC some "fidelity" to the Ag (15; and T. Sornasse, data not shown).

Our results are in accordance with previous in vitro data showing that DC are required for the development of T-dependent antibody response by mouse and human lymphocytes in vitro (26, 27). In vivo, a number of experiments have shown that the B cell is the initiating APC in peripheral lymph nodes (28–30), whereas other data suggest that non-B cells, "professional" APC, are required to initiate an

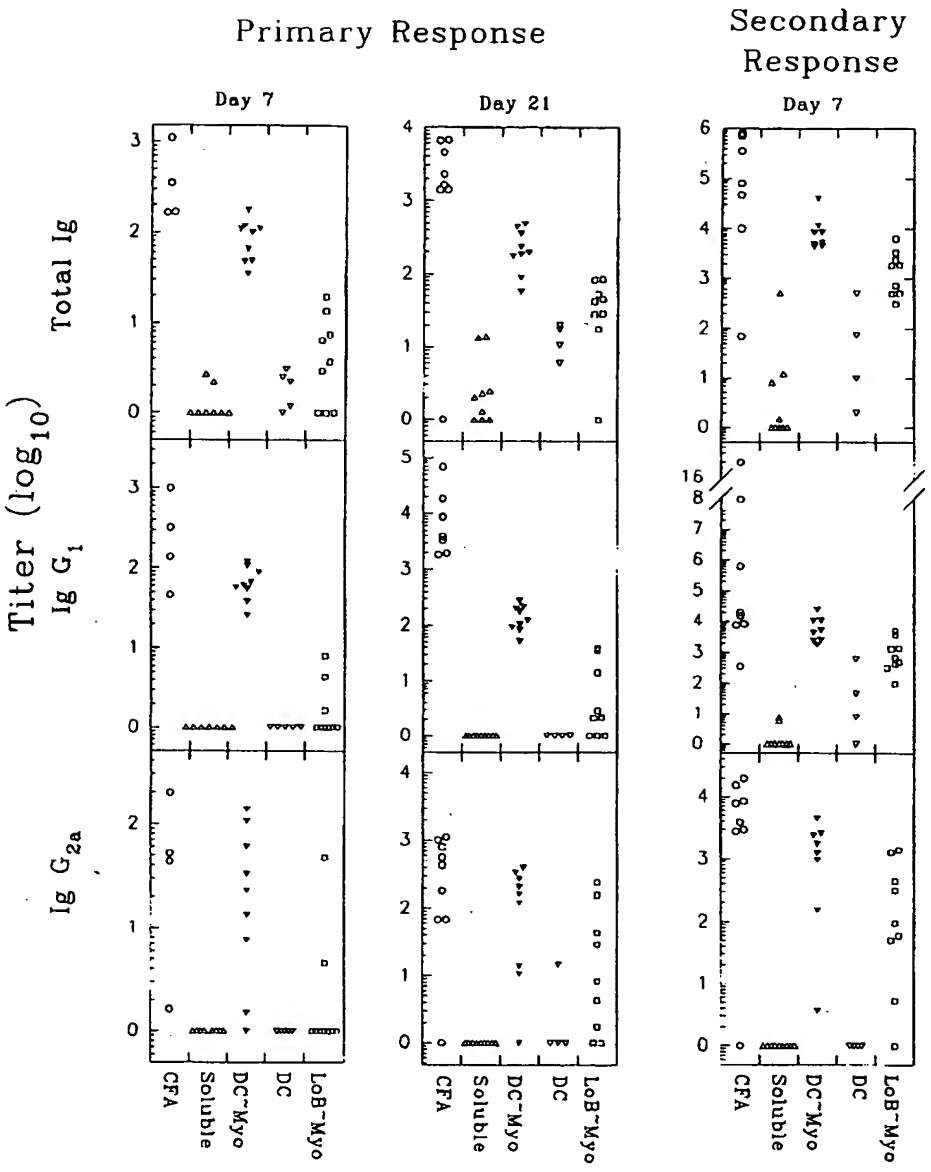


Figure 3. Isotype analysis of myoglobin-specific primary and secondary responses. Mice were injected on day 0 with either antigen-pulsed DC (*DC*~Myo), unpulsed DC (*DC*), or antigen-pulsed low-density B cells (*LoB*~Myo). All groups of mice received 100 μg of soluble antigen on day 5. Control mice include one group that was injected with 100 μg of myoglobin emulsified in CFA and one group that only received 100 μg of soluble Ag on day 5. Total specific response (*Total Ig*), as well as specific antibodies of IgG1 or IGg2a isotypes, were measured as described in Materials and Methods.

anti-SRBC primary immune response (31). Our data emphasize the main role of DC in initiating primary responses in vivo.

Low-density B cells, purified from the same low-density floating fraction as DC, very efficiently present myoglobin to T cell hybridoma in vitro. Nevertheless, they only induce a weak primary B cell response in vivo as compared with DC. However, the antibody response induced by the injection of low-density B cells is significantly higher than the response observed after injection of soluble antigen. Thus, low-density B cells may contain a population of cells able to stimulate resting T cells and initiate a humoral response. These cells could be activated B cells, or alternatively could be contaminating DC, since we detect ~1–2% of DC in the preparation (data not shown).

When primed mice were boosted with soluble Ag, a secondary response of greater amplitude is observed (Figs. 3 and 4). These results demonstrate that priming with Ag-pulsed DC elicits a memory response.

The effect of DC on antigen-specific responses most likely involves helper T lymphocytes which, in turn, recognize MHC-compatible, antigen-stimulated B cells recirculating in the vicinity. Optimal sensitization of T cells requires two steps: an antigen-specific step, the occupancy of the TCR by Ag + MHC, and an Ag nonspecific step, the costimulatory signal delivered by the APC (32, 33). IL-1 seems to be a requisite costimulator for the growth of selected $CD4^+$ Th2 clones, whereas optimal Th1 responses require a costimulatory signal that could be the murine B7 (34). Since splenic DC do not appear to secrete or express IL-1 (35), and since low-density splenic APC can replace the costimulator for Th1

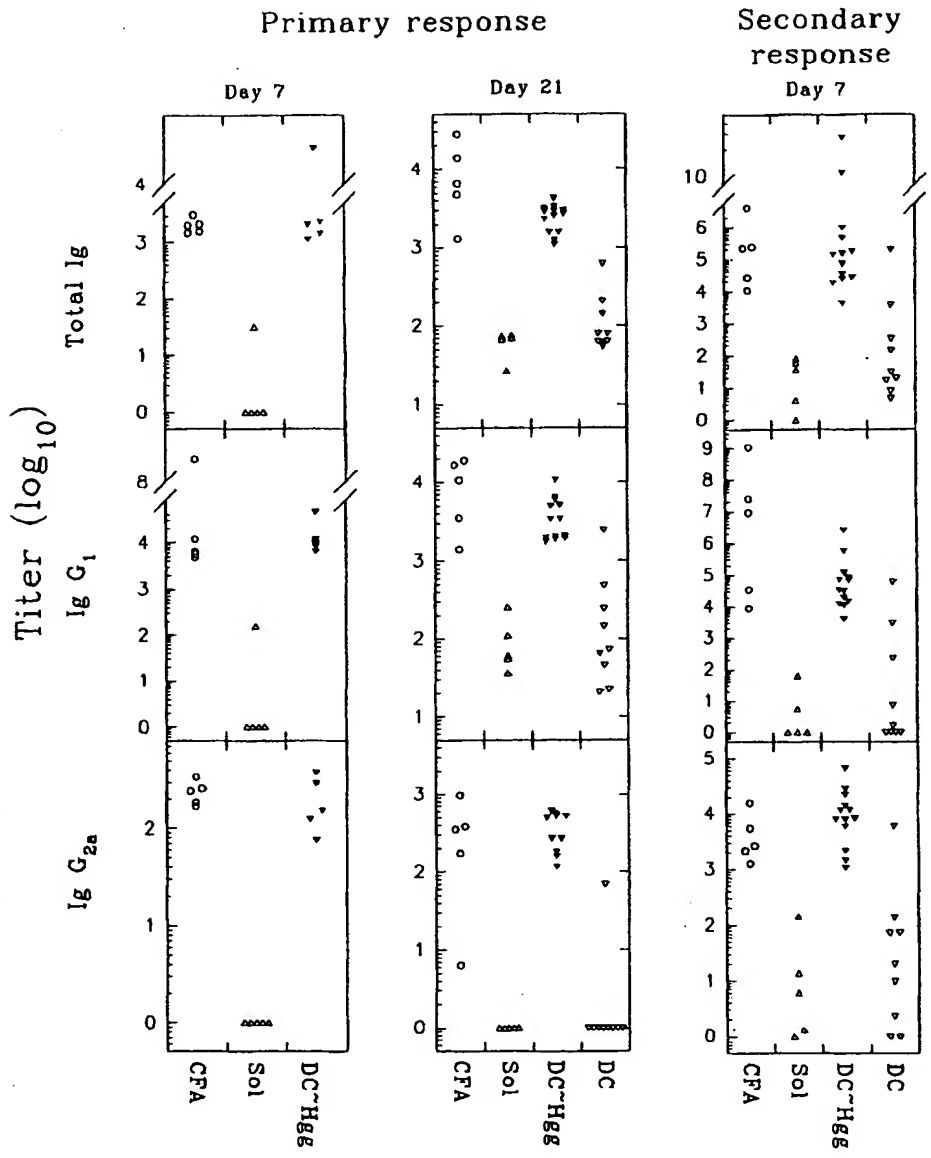


Figure 4. Isotype analysis of HGG-specific primary and secondary responses. Mice were either untreated (*Sol*), injected with antigen-pulsed DC (*DC~HGG*), or with unpulsed DC (*DC*). All groups were boosted with 100 μg of soluble antigen 5 d later. A group of mice received 100 μg of HGG emulsified in CFA (*CFA*). Total specific response (*Total Ig*), as well as specific antibodies of IgG1 and IgG2a isotypes, were tested as described in Materials and Methods.

cells (for review, see reference 36), it is tempting to speculate that injection of Ag-pulsed DC mainly activates Th1 cells in vivo.

The presence of IgG2a in most sera tested from mice primed with Ag-pulsed DC strongly supports this hypothesis. It has indeed been shown that clones of Th1 type specifically induced Ag-specific B cells to secrete IgG2a (37). Work is in progress to characterize the lymphokine pattern produced by Ag-specific, $CD4^+$ T cells isolated from the mice primed with Ag-pulsed DC.

It is of note that the synthesis of specific antibodies of IgG2a isotype is of physiological importance since this isotype has been shown to play a central role in the elimination of antigen in vivo (38, 39).

We think that Ag-pulsed DC could instruct a T helper lymphocyte, uncommitted in its lymphokine pattern, to differentiate into a Th1 type lymphocyte. The choice between Th1 and Th2 could therefore be due to the nature of the cell that presents antigen.

In conclusion, DC may be used as a physiological adjuvant to induce cellular (15) and T cell–dependent humoral responses in vivo.

We thank Drs. M. Goldman, D. Abramowicz, and P. De Baetselier for reviewing the manuscript; and Drs. N. Romani, E. Kämpgen, F. Koch, and G. Schuler (Innsbruck, Austria) for helpful discussion. We are also grateful to Mrs. Judith Aminoff for editorial assistance; to G. Dewasme and A. Rouvroy for excellent technical assistance, and to B. Platteau and O. Denis (Université Catholique de Louvain, Woluwe) for providing useful reagents.

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Declaration Dr. Moser
US Patent Application No. 09/802,397

ATTACHMENT C
MOSER I, filed for
present appl. on 6/10/05

DECLARATION BY DR. MURIEL MOSER

I, Muriel Moser, declare as follows:

I hold a Ph.D. degree in Zoology with greatest distinction from the Free University of Brussels, Belgium. Specialized in zoology since 1977, I am heading a research group focusing on the physiology of antigen presenting cells (since 1986) at the free University of Brussels. I hold, since April 2002, a degree of "Agrégé de l'enseignement supérieur" I frequently lecture at international meetings and I am a regular reviewer for several international Journals. I author over one independent, world-wide patent application and over 50 international peer-reviewed publications in the field of cell-based immunology. I have initiated the study of murine dendritic cells at the free university of Brussels and I am experimenting since the filing date of the present patent application onwards successfully with the production of human DC/tumor hybrids/hybridomas and their use to eliminate cancer in patients. I am the past president of the French group on dendritic cells. I was appointed several times as the European expert on Immunology. I enclose my Curriculum Vitae in annex (Enclosure 1).

I am one of the inventors of the US patent application US 09/802,397 and have reviewed and understand all prior art and Office Actions of record. The present Declaration illustrates that the present invention relies on unexpected results, therefore this declaration may be helpful for the examiner in advancing prosecution.

1. The claimed subject-matter

The present invention relates to a method of producing an anti-tumor response in a mammalian subject, said method comprising administering to said subject a plurality of dendritic cell/tumor hybrids and/or a dendritic cell/tumor hybridoma. The present invention illustrates for the first time that said cells may be produced and carry characteristics of tumor cells and DCs which makes them interesting for cancer therapy.

Furthermore, the present invention proves for the first time that tumors are efficiently eliminated using said hybrids/hybridomas. In particular, I have shown in this present application that said approach may easily be followed, efficient and applicable in humans.

Before the filing of the applications whereto the above-mentioned patent application claims priority (US 09/049,502; 09/025,405; 08/625,507 and 08/414,480) nobody gave the experimental/clear proof for the generation of dendritic cells (DC)/tumor hybrids or hybridomas. In particular, nobody described hybrids/hybridomas which may be applied in animal, especially human, therapy.

The present invention further teaches that DC/tumor hybrids/hybridomas may be produced efficiently starting from proliferating dendritic cells or a dendritic cell at a more immature stage. Until now, there is no cellular characteristic or marker available which may be used to define this preferred DC-fusion partner. The only definition which may apply is that said cells may be proliferating DC or are DCs at a more immature stage.

Furthermore, in order to make DC/tumor hybrids allowable in human medicine, it is essential that no essential body-part(s) of the patient is(are) used (such as spleen). A

solution to this problem is also formulated in the present invention. The present invention suggests to use cells found in for instance bone-marrow, lymph/lymph nodes or in blood. According to the present invention said cells may be proliferating dendritic cells or a dendritic cell at a more immature stage as mentioned above. Said dendritic cells may originate from induced DC-progenitors.

The approach to make human DC/tumor hybrids/hybridomas, which is applicable in human medicine, has never been suggested before the filing of any of the US applications to which the present US patent application claims priority to. Based on the prior art it was not predictable that by using dendritic cells, or, proliferating dendritic cells or a dendritic cell at a more immature stage, as described in the present application, hybrids/hybridomas could be made having both the DC and tumor characteristics which are needed to trigger tumor elimination in a patient.

2. Non-obviousness of the subject-matter of claims 1 and 3 over Guo et al. (1994) in view of Somasse et al. (1992)

I respectfully disagree with the assertion in the outstanding Office Action that that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the dendritic cells taught by Somasse et al. (1992) for the B cells of Guo et al. (1994) as the APC fusion partner in producing a dendritic cell/tumor cell hybrid, and, that it would be obvious to administer said product to a subject for production of an anti-tumor response.

In my opinion, based on said documents it is not predictable that such hybrids could be made and which characteristics said hybrids would carry. Furthermore, I am convinced that the approach of Guo for making his hybrids may not be followed to produce hybrids which may be used for human applications. As the method of making said hybrids (and thus also the starting cells) are different, it is clear that the resulting hybrids will be different. These different aspects are discussed in the paragraphs below.

The difference between the present invention and the teachings of Guo and Somasse lays thus in the definition of the hybrids AND in their use.

a/ The feasibility of making DC/tumor hybrids is not predictable

Changing the fusion partner of the tumor cell, as with the B cell of Guo et al. (1994), to another antigen-presenting cell does not allow one of skill in the art to predict the outcome of such an experiment. As mentioned in the discussion section of Carbone et al. (1988, see copy in annex (Enclosure 2), p 1374, first column, second paragreph, l.10-12), extinction or loss of expression of tissue specific traits after fusion of dissimilar cells is a well-established phenomenon (Lewin 1980; Killary and Fournier 1984). This negative regulation is not specific for cell fusions but was also observed for incoming genes (Clough et al. 1982; Palmiter et al. 1982; Gautch and Wilson 1983; Groffen et al. 1983; Hardies et al. 1983; Manor 1985; Humphries et al. 1985; Dyson et al. 1985) (p 1374, second column, second paragraph, l.1-7 of Carbone, et al.). Methylation has been shown to be a commonly used system to regulate expression within the cell; it has even been shown to be used for the natural regulation of certain T-cell surface molecules (Richardson et al. 1986) (p 1374, first column, second paragraph, l.10-15 of Carbone, et al.).

Only after extensive experiments carried out by my group it was possible to conclude that fusing tumor cells with dendritic cells as described in the present application can result in the generation of hybrids/hybridomas that carry tumor antigenic markers combined with various dendritic specific markers. The success of this fusion could not have been predicted by the prior art, and in fact, there would be sufficient reasons for one of ordinary skill in the art to believe that such a fusion of two dissimilar cells would not work, based on the above-described phenomenon of loss of expression of tissue specific traits after fusion. One of ordinary skill in the art would have taken a very cautious attitude and would not have predicted a successful outcome for this experiment until it had been demonstrated.

In this respect, it should be recognized by the Examiner that experimental evidence for the possibility to produce an anti-tumor response in a subject comprising administering dendritic cell/tumor cell hybrids/hybridomas was given for the first time in the parent of the present U.S. patent application. To reason that the outcome of the present experiments would have been predictable in advance or would be obvious is only possible when one reasons with hindsight. It is clear from the reasons cited above that the skilled person would not have reasoned in this way and would not have predicted any outcome of such an experiment before it had been proven that it works.

I hereby respectfully submit that it was not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the dendritic cells taught by Somasse et al. (1992) for the B cells of Guo et al. (1994) as the APC fusion partner in producing a dendritic cell/tumor cell hybrid. Therefore I respectfully submit that the administration of the hybrids/hybridomas of the present invention to a patient in order to produce an anti-tumor response in said patient is not obvious over Guo et al. (1994) in view of Somasse et al. (1992).

b/ The approach of Guo cannot be followed to produce hybrids/hybridomas for in vivo treatments of animals (humans)

Further, in support of the inventive step of especially claims 1 and 3, I wish to point towards the impossibility to use the method used in Guo et al. (1994) for the production of hybrid cells for animal (especially human) applications.

The method according to Guo et al. (1994) involves the use of B cells as fusion partners of the tumor cells. Said B-cells were recovered from the spleen of rats earlier injected with with soluble antigen in complete Freund's adjuvant, which cannot be applied in humans. In addition, if immunizations are done without Freund's adjuvant, the outcome of the B cells remains unpredictable in individual animals and it is expected to be unpredictable in individual human patients.

Furthermore, the approach followed by Guo does not allow multiple booster applications. In this respect I wish to stress the difference between the method used to generate hybridomas and the method used in Guo et al. (1994). In the present invention hybridomas are selected by growing them in selection medium. Unfused immortalized cell lines are killed by the exposure to a drug. In the description of the present application the use of the HAT (hypoxanthine-aminopterin-thymidine) selection medium is illustrated. After selection, the hybridoma can be cultured when needed and used for multiple booster vaccinations. This is a major advantage compared to the strategy used by Guo et al. (1994) where fusions are made and immediately used for treatment without making them immortalized;

each treatment needs a separate fusion step where variability in cell population between booster applications can be generated.

A further advantage of the DC/tumor hybrids/hybridomas of the present invention over the hybridomas of Guo et al. is that no essential body parts are needed as start material, that the fusion partners are easy to isolate, that it allows the treatment of a subject using cells which are compatible with its immune system and that the approach of the present invention allows to produce a product for which a guarantee of its composition and quality may be given. All these aspects are discussed in detail in section 3 (see below).

3. Non-obviousness of the subject-matter of claims 19-26 over Guo et al. (1994) in view of Sornasse et al. (1992)

I am of the opinion that the note of the Examining Division in respect of the fact that the spleen cells of Guo et al. (1994) and Sornasse et al. (1992) would also comprise an isolated DC as well as the only two known murine subtypes of DC (i.e. myeloid and lymphoid, both of which are derived from bone marrow) is inappropriate.

I would like to point out that both myeloid and lymphoid DC may be considered as isolated DCs.

Claims 19-20 and 47-50

It is true that all DCs found *in vivo* (thus also from spleen) are originally bone marrow derived. However, what the present application teaches is different. The present application illustrates that DC-cells derived from bone marrow, lymph/lymph nodes, blood or other tissues are a better alternative to spleen cells to start the production of the hybrids of the present invention.

I confirm hereby that my group has the experience that a DC-preparation from spleen is not a good start population to aim for the production of DC/tumor hybrids/hybridomas. Indeed I have the experience that primary cultured DCs (proliferating DCs) are preferentially needed (e.g. cultured from bone marrow cells) to produce said hybridomas; using primary DCs (non-proliferating DCs; e.g.isolated from spleen) will not result in the efficient and reproducible production of DC/tumor hybrids/hybridomas. I have the experience that when using preparations of spleen cells mainly hybrids may be formed between non-DCs (for example T cells) and tumor cells even if the DC population in said preparation is dominant. Consequently, I am of the opinion that starting from mouse splenic cells is extremely doubtful that a real DC/tumor hybridoma can be obtained.

In addition, bone narrow, lymph/lymph nodes and blood may contain DC-precursors or intermediates between monocytes and DCs. Said monocytes may be used to further differentiate into proliferating intermediates between monocytes and DCs or into proliferating DCs before the production of the hybrids/hybridomas of the present invention. These intermediates or precursor cells are not present in tissues such as spleen.

In the section below I further explain that the method applied by Guo to produce the DC/tumor hybridomas may not be applied in medicine (humans or animals) and thus has no industrial value.

A further optimization of DC/tumor hybrids of the present invention, is that said hybrids/hybridomas may be applied in medicine (human and animal). Indeed, the DC used to make the hybrids/hybridomas of the present invention are purified from cells without the need for isolating essential organs/cells from said mammals such as spleen. Using spleen cells, as used in the method of Guo et al, 1994, inherently results in the killing of the organism from which said cells are retrieved. Contrarily, the use of DC fusion partners derived from blood, lymph/lymph nodes or bone marrow as proposed in the present invention allows to keep the organism, from which said cells are isolated, alive.

Furthermore, a patient may be treated with cells derived fom his own body making the cancer treatment compatible with his immune system. A higher efficiency of the therapy is therefore also expected.

In summary, the use of DC-fusion partners purified from blood, lymph/lymph node or bone marrow cells allows a more efficient production of the hybrids/hybridomas of the present invention. In addition, these bone marrow and blood cells are easy to isolate and allow a better approach for human applications. I also refer to the arguments given in section 2/b (above).

Claims 21-26

I hereby explain the cellular origin of existing DCs in animals.

DC precursor cells from bone marrow may be considered as the stem cell from which DC myeloid precursor cells (e.g. monocytes) and DC lymphoid precursor cells may differentiate. Both precursor cells may give rise to differentiated, also called mature DCs; myeloid and lymphoid DC respectively.

Mature myeloid and lymphoid DCs may be found in specific tissues, such as spleen, but may also be present in for instance blood. Precursor cells of myeloid or lymphoid origin may be present all over the body. However, it is accepted in the scientific literature that these are not present in specific tissues, such as spleen.

Most stem cells (bone marrow) or precursor cells are present in a resting state. This means that these are non-proliferating. However, proliferation may be induced (in vivo or in vitro) in said cells.

I have the experience that the hybrids/hybridomas of the present invention may be made with high efficiency when differentiating blood, lymph/lymph nodes or bone marrow DC-precursors or proliferating DCs (before they enter in the resting state) are used. Said cells are therefore considered as preferable fusion partner for the production of the hybrids/hybridomas of the present invention (new claims 29-46).

4. Non-obviousness of the subject-matter of claims 5-10 over Guo et al. (1994) in view of Sornasse et al. (1992) and in further view of U.S. Patent 5,851,756.

As explained above, skilled person may not derive from Guo et al. and Sornasse that such a DC/tumor hybrid may be formed.

In addition, I respectfully disagree that based on the '756 patent one of ordinary skill in the art at the time of the invention would have been motivated to induce DC-characteristics (using GM-CSF) in DC/tumor hybrids/hybridomas before using said fused cells in anti-tumor treatments.

This is non-obvious based on the following reasons:

1/ The induction in the method of the present invention is performed *after* the hybrid formation, the DCs corresponding to the DCs of the '756 patent do not exist anymore.

2/ The DCs of the '756 patent are different from the hybrids of the present invention. It is not obvious that such a hybrid would behave like the isolated DC cell of the '756 patent.

3/ The induction allows to induce the expression of DC characteristics, not to increase the number of DC cells in blood as taught in the '756 patent and explicitly repeated by the Examining Division.

5. Non-obviousness of the subject-matter of claims 11-14 over Guo et al. (1994) in view of Somasse et al. (1992) and in further view of U.S. Patent 5,637,483.

I respectfully disagree that based on the '483 patent an ordinary skilled in the art at the time of the invention would have been motivated to irradiate the hybrids/hybridomas of the present invention to prevent proliferation.

According to the '483 patent, the irradiation of the tumor vaccine is presented as an essential step. I hereby refer to the claims of said patent and to especially example 6 of said patent. In said example, vaccination studies using life transduced tumor cells are discussed. In said experiment (column 14, l.48-50) it is explicitly mentioned that all tumor cells, except the IL-2 secreting tumor cells, resulted in tumor formation. It is obvious that life-tumor-cells would never be accepted as vaccine in human therapy.

Contrarily, in the present invention it is suggested that said hybrids/hybridomas may be irradiated. However, said irradiation is not an essential step in the production of said vaccine. Indeed, the hybrids/hybridomas of the present invention are fusions between tumor cells and DC cells. They may have predominant tumor characteristics, predominant DC characteristics, or an equal distribution of both characteristics. For cells with especially predominant DC characteristics, said irradiation may have a more negative effect on the anti-cancer therapy. The present invention teaches explicitly that also living hybrids/hybridomas elicited an anti-tumor immune response (paragraph [0100], l.9-12 of the application as published). Therefore, said cells have predominantly DC characteristics and do not need the irradiation step as proposed in the '483 patent before it is used in the anti-cancer therapy.

Said irradiation should be considered as an essential step in the approach of the '483 patent, and is optional in the method of the present invention. This proves that the hybrids of the present invention are clearly different from the tumor cells of the '483 patent, and that the characteristics of said hybrids are not predictable based on the tumor cells of the '483 patent.

I am convinced that the teachings of the present invention and the '756 patent are not comparable.

Declaration Dr. Moser
US Patent Application No. 09/802,397

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true.

Signed this 7 day of November, 2003

Muriel Moser

ATTACHMENT 1

"MOSER I" Declaration as filed in related application no. 09/951,849

Declaration Dr. Moser
US Patent Application No. 09/951,849

DECLARATION BY DR. MURIEL MOSER

I, Muriel Moser, declare as follows:

I hold a Ph.D. degree in Zoology with greatest distinction from the Free University of Brussels, Belgium. Specialized in zoology since 1977, I am heading a research group focusing on the physiology of antigen presenting cells (since 1986) at the free University of Brussels. I hold, since April 2002, a degree of "Agrégé de l'enseignement supérieur" I frequently lecture at international meetings and I am a regular reviewer for several international Journals. I author over one independent, world-wide patent application and over 50 international peer-reviewed publications in the field of cell-based immunology. I have initiated the study of murine dendritic cells at the free university of Brussels and I am experimenting since the filing date of the present patent application onwards successfully with the production of human DC/tumor hybrids/hybridomas and their use to eliminate cancer in patients. I am the past president of the French group on dendritic cells. I was appointed several times as the European expert on Immunology. I enclose my Curriculum Vitae in annex (Enclosure 6).

I am one of the inventors of the US patent application US 09/951,849 and have reviewed and understand all prior art and Office Actions of record. The present Declaration illustrates that the present invention relies on unexpected results, therefore this declaration may be helpful for the examiner in advancing prosecution.

1. The claimed subject-matter

The present invention relates to a plurality of dendritic cell/tumor hybrids and/or hybridomas which induce an anti-tumor response when applied to a patient in need of an anti-tumor treatment by reducing the number of tumor cells in said patient corresponding to the tumor cell in said hybrids. The present invention illustrates for the first time that said cells may be produced and carry characteristics of tumor cells and DCs which makes them interesting for cancer therapy.

Before the filing of the applications whereto the above-mentioned patent application claims priority (US 09/049,502; 09/025,405; 08/625,507 and 08/414,480) nobody gave the experimental/clear proof for the generation of dendritic cells (DC)/tumor hybrids or hybridomas. In particular, nobody described human hybrids/hybridomas which may be applied in human therapy.

The present invention further teaches that DC/tumor hybrids/hybridomas may be produced efficiently starting from proliferating dendritic cells or a dendritic cell at a more immature stage. Until now, there is no cellular characteristic or marker available which may be used to define this preferred DC-fusion partner. The only definition which may apply is that said cells may be proliferating DC or are DCs at a more immature stage.

Furthermore, in order to make DC/tumor hybrids allowable in human medicine, it is essential that no essential body-part(s) of the patient is(are) used (such as spleen). A solution to this problem is also formulated in the present invention. The present invention suggests to use cells found in for instance bone-marrow, lymph/lymph nodes or in blood. According to the present invention said cells may be proliferating dendritic cells or a dendritic cell at a more

Immature stage as mentioned above. Said dendritic cells may originate from induced DC-progenitors.

The approach to make human DC/tumor hybrids/hybridomas, which is applicable in human medicine, has never been suggested before the filing of any of the US applications to which the present US patent application claims priority. Based on the prior art, it was not predictable that by using dendritic cells, or, proliferating dendritic cells or a dendritic cell at a more immature stage, as described in the present application, hybrids/hybridomas could be formed having both the DC and tumor characteristics which are needed to trigger tumor elimination in a patient.

2. Teaching of Peters, 1981 and novelty of claimed subject-matter over said prior art document

Although the title of Peters (1981) seems to suggest the production of DC/tumor hybridomas, I am of the opinion that this document does not teach the skilled reader that said hybridomas are actually made, nor, does it not allow the person skilled in the art to repeat the production of said alleged hybridomas. Different aspects of said prior art document are discussed in the paragraphs below.

That Peters (1981) does not disclose the fusion of a real DC with a tumor cell may be asserted based on four facts:

1/ the start population for the DC-fusion partner is a mixed cell population
2/ the hybridomas obtained by Peters are not well characterized
3/ the results of Peters are not convincing, not-enabling and not reproducible
4/ Peters is not the founder of the DC/tumor hybridoma technology

1/ the start population for the DC-fusion partner is a mixed cell population

For the preparation of the CBA mouse DCs, the author refers to the abstract of Peters (1980) (see copy in Enclosure 1). Said one page abstract describes the isolation of murine adherent cells from mouse spleen (112) and the isolation of human adherent cells from periferal human blood (113). As Peters (1981) refers to the preparation of murine cells (see copy in Enclosure 1), I am convinced that a person skilled in the art would only focus on the first abstract (112).

The (112) abstract describes a method for the preparation of spleen cells. According to this abstract said cell preparation still contains a small fraction of esterase-positive adherent cells which are non phagocytic and show mitogenic cooperation with T cells (see lines 8-9 of Peters (1980)). Said preparation is depleted of macrophages (line 7 of Peters (1980)) but contains according to my scientific experience a large number of other types of cells. This thought is confirmed by the fact that the criterion of adherence for separating these cells (DC) from T cells is not sufficient (see lines 13-14 of Peters (1980)). Consequently, Peters himself agrees that he starts from a mixed cell population. Consequently, he already suggests that his cell hybridomas may be the result of the fusion of tumor cells with cells other than dendritic cells.

The isolated spleen fusion partners of Peters (1981) were found to show little or no phagocytosis, to show adherence to hydrophobic surfaces and to be weakly positive for unspecific esterase (Peters, 1981, lines 6-7). Most splenic cells, except macrophages, would show non phagocytic activity and be weakly positive (or negative) for unspecific esterase. Therefore, the skilled reader is persuaded that a mixed cell population is used to

make the cell hybrids of Peters (1981). Consequently, the skilled reader is persuaded that said hybridomas may be the result of the fusion of tumor cells with cells other than dendritic cells.

I confirm that the present application gives for the first time a clear proof that DC/tumor hybrids/hybridomas can be made. In addition, the present application discloses for the first time a further optimization for the production of said DC/tumor hybrids.

I further confirm hereby that my group has the experience that a DC-preparation from spleen is not a good start population to aim for the production of DC/tumor hybrids/hybridomas. Indeed I have the experience that primary cultured DCs (proliferating DCs) are preferentially needed (e.g. cultured from bone marrow cells) to produce said hybridomas; using primary DCs (non-proliferating DCs; e.g.isolated from spleen) will not result in the efficient and reproducible production of DC/tumor hybrids/hybridomas. I have the experience that when using preparations of spleen cells mainly hybrids may be formed between non-DCs (for example T cells) and tumor cells even if the DC population in said preparation is dominant. Consequently, I am of the opinion that starting from mouse splenic cells (as described in Peters, 1981) it is extremely doubtful that a real DC/tumor hybridoma could be obtained. Only 14 different cell lines were isolated. In addition, as said production is doubtful a skilled person would never apply the teaching of Peters to make hybridomas for clinical applications.

2/ the obtained hybridomas are not well characterized

In my opinion, the obtained hybridomas in Peters (1981) were not clearly characterized. There is no real confirmation that indeed DC/tumor cells hybridomas were formed. This is clear from different aspects of Peters (1981) and discussed in the paragraphs below. In addition, the existence of tumor/tumor and DC/DC hybrids may not be excluded from this mixture.

As Peters uses the wording "hybridomas approaching the DC phenotype" (Peters (1981) lines 9-10) it is clear for the skilled reader that the author at that moment was not convinced of the real nature of the fusion partner of the tumor cell within the hybridoma.

Further, it is not clear for the skilled reader what the wording "the retained selected and combined DC properties" (lines 4-5) of Peters (1981) implies.

In Peters (1981), it is mentioned that the lines differ markedly in their *morphology* (line 13). However, no further details are given for the skilled reader.

In addition, one cell line is said to stain positive with *anti-Ia serum* (lines 11-12, 15-16). As B cells and macrophages express intermediate or low levels of class II MHC molecules, respectively, whereas DC express high levels, it cannot be excluded that said hybridomas are the result of the fusion of cells other than DCs. Consequently, the skilled reader will be aware that the presence of Ia antigen cannot be interpreted as a proof of the presence of DC characteristics. In addition, the plasmacytoma cell, which is used as cancer cell to make the hybridoma in Peters (1981), is a B cell. It is known that B cells may carry this class Ia antigen. Therefore, I am of the opinion that showing the presence of this antigen may even not be considered as a proof for the presence of characteristics of cells other than tumor cells in the resulting hybridoma of Peters (1981).

MM Peters (1981) describes that three lines exhibit a strong *inductive capacity on T lymphocyte growth* in the absence and/or presence of mitogens (lines 14-15). To my experience, said

induction may result from other causes than from the presence of DC characteristics on said hybridomas.
1/ It is not clear from Peters (1981) if the T cells used are naïve T cells or activated T cells. If said T cells are already in an activated state, they may be stimulated by tumor cells "as such". It is only when naïve T cells are used that the induction of T cells may be caused by the presence of DC characteristics; proving that the hybridoma carries DC characteristics.
2/ Lymphosarkoma cells from AKR mice (line 8) or plasmocytoma cells from BALB/C mice (line 9) were used to fuse with (DC)-cells from CBA mouse (line 5). This means that semi-allogeneic hybridomas are formed to study the proliferation of T cells. Due to the presence of allogeneic characteristics it is possible that hybridomas induce the T cells based on the tumor characteristics and not due to the presence of the DC characteristics. The tumor cells "as such" may have the capacity of inducing the T cell proliferation. There is no control included in the experiments of Peters (1981) excluding these possibilities.
Therefore, I am of the opinion that the stimulation of the T cell proliferation is not a proof of the presence of DC characteristics in the hybridomas as described in Peters (1981).

3/ the results of Peters are not convincing, not enabling and not reproducible.

No experimental details are given to make and characterize the hybridomas of Peters (1981), making the reproducibility of the results presented not possible for a person skilled in the art. Therefore, the method to produce hybridomas as disclosed in Peters (1981) should be considered not only as non-convincing but also as non-enabling. Therefore, a skilled person is not able to repeat the experiments of Peters in order to check if real DC/tumor hybridomas can be made.

Furthermore, Peters (1981) mentions that 14 different lines (slow growing!) could be isolated, three of them exhibiting a strong capacity on T lymphocyte growth, whereby only one stains positive for anti-Ia serum (lines 11 to 16). As there is only a low frequency for recovering said type of hybridoma it becomes clear that the production of said hybridoma is a rare event. In addition, it is clear from the above-mentioned discussion that in Peters (1981) no proof is given that actually a real DC/tumor hybridoma was made. As said abstract also does not give the essential experimental details, the skilled reader would not be convinced that Peters (1981) teaches the production of DC/tumor hybridomas and would thus not be encouraged to repeat the experiments as presented in Peters (1981).

4/ Peters is not the founder of he DC/tumor hybridoma technology

a. Peters is not considered by third parties as the founder of the DC/tumor hybridoma technology

After the priority date of the present patent application, many articles have been published which confirm now that fusions of DCs with tumor cells are indeed feasable resulting in the generation of hybrids carrying anti-tumoral characteristics (Gong et al. (1997), Cao et al. (1999), Gong et al. (1998); Wu et al. (1998); Wang et al. (1998), Kugler et al. (2000), Gong et al. (2000a) and Gong et al. (2000b)). These articles, published by others after the priority date of the present invention, also stress the novelty and inventiveness of the presently claimed subject-matter. Copies of these articles are enclosed for the Examiner's convenience (Enclosure 1). In none of these articles, Peters (1981) nor any other article published by the group of Peters, was cited as one of the first articles describing the production of DC/tumor hybrids.

MM

Interestingly, none of these recent publications cite the abstract of Peters (1981). Contrarily, the article published by our group (Lespagnard et al. 1998) has been cited frequently. This proves that also other research groups do not recognize the group of Peters, but rather our group, as being one of the founder groups in respect of the production of DC hybrids/ hybridomas.

b. Peters does not consider himself the founder of the DC/tumor hybridoma technology

In order to evaluate the scientific value of the Peters (1981) abstract, I searched for later publications by Peters which normally should follow the release of the cited Peters (1981) abstract. However, following the abstract, Peters did not publish an article that confirms the content of the Peters (1981) abstract. I followed closely from the beginning the development of the DC/tumor hybrid/hybridoma field and have contacts with colleagues in the same research area. Until the citation of said abstract in the present examination procedure I was not aware of the existence of said publication, nor was I aware of the existence of his research group.

I have enclosed a copy of documents which can be retrieved when searching for published data of "peters jh" in respect of "dendritic cells" (Enclosure 2). From this search it is clear that Peters's research group only started to publish on the production and the use of DC/tumor hybrids and hybridomas from 2001 on [Soruri et al. (2001), Krause et al. (2002) and Haenssle et al. 2002). So he only recently joined this research field. In addition, it should be stressed that no peer-reviewed publication proving the existence of DC/tumor cell hybrids/hybridomas followed in the years after the 1981 abstract of Peters.

In the introduction of the Soruri et al. (2001) article it is mentioned by the authors (amongst which Peters) that DC/tumor cells were previously found to eliminate established metastatic diseases. Reference is made towards Gong et al. (1997, Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med 3:558.), Lespagnard et al. (1998, Dendritic cells fused with mastocytoma cells elicit therapeutic antitumor immunity. Int J Cancer 76:250) and, Wang et al. (1998, Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. J. Immunol. 161:5516). All said documents cited by Soruri et al. are published after the priority date of the present patent application. In addition, it is clear from said citation that Peters does not consider himself as being the founder of the concept of the DC/tumor fusions as Peters does not refer to articles published by his own group in this respect. In addition, he does not even mention his abstract published in 1981, confirming that the content of said abstract may not be considered as being relevant/convincing for the generation of DC/tumor hybrids/hybridomas. He cites his own articles only in respect of side aspects of said concept (Jung et al. (1995) Interleukin-4 and Interleukin-5 are rarely co-expressed by human T-cells. Eur. J. Immunol. 25:2413 and Soruri et al. (1998) Specific autologous anti-melanoma T cell response *in vitro* using monocyte-derived dendritic cells. Immunobiology 198: 527). The publication of Lespagnard et al. belongs to my research group. Therefore, the group of authors amongst which is Peters admit that my research group may be considered as one of the founders of the production of DC/tumor hybrids/hybridomas.

Also the article Krause et al. (2002) only refers in their introduction (p.422, first column, first paragraph, l.12-18) to the prior articles Guo et al (1994), Gong et al. (1997), and Soruri et al. (2001) in respect of the production of DC/tumor hybrids. Peters (1981) nor any other early data published by Dr. Peters are mentioned in this respect.

MM The paragraphs above elaborate on the fact that Peters (1981) does not disclose the fusion of a real DC with a tumor cell. In the section below I further explain that the method applied

by Peters to produce the DC/tumor hybridomas may not be applied in medicine (humans or animals) and thus has no industrial value.

A further optimization of DC/tumor hybrids of the present invention, is that said hybrids/hybridomas may be applied in medicine (human and animal). Indeed, the DC used to make the hybrids/hybridomas of the present invention are purified from cells without the need for isolating essential organs/cells from said mammals such as spleen. Using spleen cells, as used in the method of Peters (or Guo et al, 1994), inherently results in the killing of the organism from wich said cells are retrieved. Contrarily, the use of DC fusion partners derived from blood or bone marrow as proposed in the present invention allows to keep the organism, from which said cells are isolated, alive.

Furthermore, a patient may be treated with cells derived fom his own body making the cancer treatment compatible with his immune system. A higher efficiency of the therapy is therefore also expected.

In addition, as said DC fusion partners may be isolated from blood, lymph/lymph nodes and bone marrow, they can easily be isolated from a mammal.

Conclusion

As a conclusion I agree that the idea of making DC/tumor hybrids is mentioned in the title of Peters (1981). However, I find it by no means proven for the skilled reader that the hybridomas mentioned to have been produced in Peters (1981) are indeed real DC/tumor hybridomas. Consequently, Peters (1981) gives no clear proof of the possibility to generate DC/tumor hybridomas.

In addition, the method to produce the hybridomas as disclosed by Peters (1981) may not be applied to make DC/tumor hybridomas allowable in animal (human) medicine. Peters (1981) does not allow a skilled person to make spleen/tumor hybridomas with reproducible characteristics. It is not allowable that compositions are used in medicine for which no guarantee of its composition and quality may be given.

Furthermore, we also suggested for the first time that DC purified from blood, bone marrow or lymph/lymph nodes are better start material to produce DC/tumor hybrids/hybridomas. This is also not disclosed or suggested in Peters (1981).

We also suggest for the first time that the dendritic fusion partner, used to produce DC/tumor hybrids/hybridomas, should be preferentially proliferating cells or dendritic cells at a more immature stage. This is not disclosed or suggested in Peters (1981).

Only by following the approach of the present invention a skilled person may be convinced that DC/tumor hybrids may be formed.

In this respect, I do not find it appropriate that the Examiner would cite Peters (1981) as a novelty destroying document for new claims 1, 5, 32, 33, 34, 37, 38, 39, 58, 59, 60, and 61 (previous claims 1-3, 5-7, 9 and 32-41). In addition I am convinced that also the subject-matter of new claims 42 to 55 is new and inventive over Peters (1981).

3. Novelty of the claimed subject-matter over U.S. Patent No. 4,711,842

HM I would like to point to the fact that, as previously mentioned by the Examiner, that monocytes and DCs are different cells, each having their particular characteristics.

Therefore, I am convinced that the hybrids/hybridomas of the '842 patent are different from the hybrids/bybridomas of the present invention.

As said characteristics are so different, it is not evident for a skilled person to change the monocytes of the '842 by the DC of the present invention and predicting the possibility that said hybrid/hybridomas can be made. I refer hereby to the discussion of section 4 in respect of Guo et al. (see below).

Additionally, I would also like to point the Examiner's attention to the fact that the present application further teaches that not monocytes, but cells *derived* from monocytes, are used to produce the hybrids and/or hybridomas of the present invention.

Monocytes are precursors of dendritic cells which may be used to make the hybrids/hybridomas of the present invention. The present application teaches in particular that the choice of the moment (cellular state, dividing) at which the DC cell is taken to produce the hybrid/hybridoma of the present invention is important.

The monocytes which are suggested as fusion partner by the '842 patent are resting/non-differentiating cells and do not proliferate. This is clearly indicated by the '842 patent [on for instance col.6, l.15 and l.21] that the proliferating capacity of said hybrids is an unexpected feature. Indeed, the hybrids of US4,711,842 are the result of fusing non-proliferating/non-dividing cells (such as macrophages or monocytes) with proliferating tumor cells. This stands in contrast with the dividing DCs used as preferred fusion partner for the production of the hybrids/hybridomas of the present invention. Not only the proliferative capacity of said cells is introduced, but a whole battery of characteristics change in said cells.

Based on US4,711,842 a skilled reader may not derive the teaching of the present invention. During the differentiation many different cellular characteristics change, therefore both cell types need to be considered as different. Consequently, the hybrids/hybridomas made using monocytes or using proliferating dendritic cells or dendritic cells at a more immature stage (except monocytes) are different.

4. Non-obviousness of the claimed subject-matter over Guo et al. (1994) in view of Sornasse et al. (1992)

4.A. Non-obviousness

I respectfully disagree with the assertion in the outstanding Office Action that that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the dendritic cells taught by Sornasse et al. (1992) for the B cells of Guo et al. (1994) as the APC fusion partner in producing a dendritic cell/tumor cell hybrid.

In my opinion, based on said documents it is not predictable that such hybrids could be made with characteristics of both dendritic cells and tumor cells. Furthermore, I am convinced that the approach of Guo for making his hybrids may not be followed in order to produce hybrids which may be used for human applications. As the method of making said hybrids (and thus also the starting cells) are different, it is clear that the resulting hybrids will be different. These different aspects are discussed in the paragraphs below.

MM

Declaration Dr. Moser 8
US Patent Application No. 09/951,849

1/ The feasibility of making DC/tumor hybrids is not predictable

Changing the fusion partner of the tumor cell, as with the B cell of Guo et al. (1994), to another antigen-presenting cell does not allow one of skill in the art to predict the outcome of such an experiment. As mentioned in the discussion section of Carbone et al. (1988, see copy in Enclosure 1, p 1374, first column, second paragraph, l.10-12), extinction or loss of expression of tissue specific traits after fusion of dissimilar cells is a well-established phenomenon (Lewin 1980; Killary and Fournier 1984). This negative regulation is not specific for cell fusions but was also observed for incoming genes (Clough et al. 1982; Palmiter et al. 1982; Gautch and Wilson 1983; Groffen et al. 1983; Hardies et al. 1983; Manor 1985; Humphries et al. 1985; Dyson et al. 1985) (p 1374, second column, second paragraph, l.1-7 of Carbone, et al). Methylation has been shown to be a commonly used system to regulate expression within the cell; it has even been shown to be used for the natural regulation of certain T-cell surface molecules (Richardson et al. 1986) (p 1374, first column, second paragraph, l.10-15 of Carbone, et al.).

Only after extensive experiments carried out by my group it was possible to conclude that fusing tumor cells with dendritic cells as described in the present application can result in the generation of hybrids/hybridomas that carry tumor antigenic markers combined with various dendritic specific markers. The success of this fusion could not have been predicted by the prior art, and in fact, there would be sufficient reasons for one of ordinary skill in the art to believe that such a fusion of two dissimilar cells would not work, based on the above-described phenomenon of loss of expression of tissue specific traits after fusion. One of ordinary skill in the art would have taken a very cautious attitude and would not have predicted a successful outcome for this experiment until it had been demonstrated.

In this respect, it should be recognized by the Examiner that experimental evidence for the possibility to produce DC/tumor hybrid/hybridoma has been given for the fist time in the present U.S. patent application 09/951,849 and applications whereto priority is claimed by said application. To reason that the outcome of the present experiments would have been predictable in advance or would be obvious is only possible when one reasons with hindsight. It is clear from the reasons cited above that the skilled person would not have reasoned in this way and would not have predicted any outcome of such an experiment before it had been proven that it works.

2/ The approach of Guo cannot be followed for the in vivo treatments of animals (humans)

Further, in support of the inventive step of especially claims 1 and 5, I wish to point towards the impossibility to use the method used in Guo et al. (1994) for the production of hybrid cells for human applications.

The method according to Guo et al. (1994) involves the use of B cells as fusion partners of the tumor cells. Said B-cells were recovered from the spleen of rats injected earlier with soluble antigen in complete Freund's adjuvant, which cannot be applied in humans. In addition, if immunizations are done without Freund's adjuvant, the outcome of the B cells remain unpredictable in individual animals and it is expected to be unpredictable in individual human patients.

As said cells were also recovered from spleen we refer to the arguments described above in respect of Peters (1981).

Furthermore, the approach followed by Guo does not allow multiple booster applications. In this respect I wish to stress the difference between the method used to generate hybridomas and the method used in Guo et al. (1994). In the present invention hybridomas

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are selected by growing them in selection medium. Unfused immortalized cell lines are killed by the exposure to a drug. In the description of the present application the use of the HAT (hypoxanthine-aminopterin-thymidine) selection medium is illustrated. After selection, the hybridoma can be cultured when needed and used for multiple booster vaccinations. This is a major advantage compared to the strategy used by Guo et al. (1994) where fusions are made and immediately used for treatment without making them immortalized; each treatment needs a separate fusion step where variability in cell population between booster applications can be generated.

4.B. Myeloid and lymphoid DCs

Finally, the US-Examining Division noted that the terms myeloid and lymphoid only refer to mouse DC.

When considering human dendritic cells, it is true that nowadays the skilled person refers to myeloid and plasmacytoid DCs. Said cells may be discriminated from each other based on the expression of the CD11c receptor (see Donaghy et al, Enclosure 5). Myeloid DC are defined as CD11+, plasmacytoid are defined as CD11c- cells. A skilled person will immediately converse the DC-terminology depending on the animal considered. Consequently, the use of the terms myeloid and lymphoid in the claims does not limit the use of the hybrids/hybridomas of the present invention to applications in mice only.

Furthermore, new claims 4 and 8 specify that the dendritic cell and/or tumor cell is of human origin. However, a similar approach may be followed to produce DC/tumor hybrids/hybridomas for other animals/mammals.

In hematopoiesis, a pluripotent DC stem cell differentiates along one or two pathways, giving rise to either a lymphoid-DC stem cell or a myeloid-DC stem cell. Lymphoid- and myeloid- stem cells differentiate into DC progenitor cells (of thus different origin). The DC-progenitor cells further differentiate in mature DCs (of myeloid or lymphoid origin).

The terms lymphoid and myeloid define therefore the cellular origin but do not explain where they can be found in the body. For instance, myeloid and lymphoid precursor cells and mature DCs of myeloid and lymphoid origin may be present blood. In addition, bone marrow comprises stem cells giving rise to the different cell types. Specific tissues such as spleen only comprise mature DCs. Therefore the subject matter of for instance claims 52-54 is different from the subject-matter of claims 55-56.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true.

Signed this 3d day of november, 2003

M Moser

Muriel Moser

Annexed articles:

Cao et al. (1999). Therapy of established tumor with a hybrid cellular vaccine generated by using granulocyte-macrophage colony-stimulating factor genetically modified dendritic cells. Immunol. 4: 616-625.

Carbone et al. (1988) Remethylation at sites 5' of the murine Lyt-2 gene in association with shutdown of Lyt-2 expression. J. Immunol. 141:1369-1375.

Gong et al. (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nature medicine 3: 558-561.

Gong et al. (1998) Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice Inmmunized with fusions of dendritic and carcinoma cells. Proc. Natl. Acad. Sci. USA 95: 6279-6283.

Gong et al. (2000a) Fusions of human ovarian carcinoma cells with autologous or allogenic dendritic cells induce antitumor immunity. J. Immunol. 165: 1705-1711.

Gong et al. (2000b) Activation of antitumor cytotoxic T ymphocytes by fusions of human dendritic cells and breast carcinoma cells. Proc. Natl. Acad. Sci. USA 97: 2715-2718.

Haenssle et al. 2002. DC2002 meeting in Bamberg. poster P140.

Krause et al. (2002) The treatment of patients with disseminated malignant melanoma by vaccination with autologous cell hybrids of tumorcells and dendritic cells. J. Immunother. 25: 421-8.

Kugler et al (2000) Regression of human metastaic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nature medicine. 6: 332-336.

Lespagnard et al. (1998), Dendritic cells fused with mastocytoma cells elicit therapeutic antitumor immunity. Int J Cancer 76:250-258.

Peters (1980) Adherent cell heterogeneity (I) : non phagocytic adherent cells in mouse spleen. Immunol. 157: 261.

Peters (1981) Dendritic cell (DC) hybridoma action on T lymphocyte proliferation. Immunobiology 159 : 159 XP001000958.

Soruri et al. (2001) Ex vivo generation of human anti-myeloma autologous cytotoxic T cells by dendritic cell/myeloma cell hybridomas. Cancer Immunol. Immunother 50: 307-314.

Wang et al. (1998) Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. J. Immunol. 161: 5516-5524.

Wu et al. (1998) Treatment of hepatocellular carcinoma with the cellular tumor vaccines generated by in vitro modification of tumor cells with non gene transfer approaches. Adv. Exp. Med. Biol. 451: 283-93.

ATTACHMENT E (moser II)

DECLE55.1CP2DV

TENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Moser, et al.	) Group Art Unit: 1644
Appl. No.	:	09/802,397	)
Filed	:	March 9, 2001	)
For	:	**DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE**	)
Examiner	:	Ewoldt, Gerald R.	

SECOND DECLARATION OF DR. MURIEL MOSER UNDER 37 C.F.R. § 1.132
(MOSER II)

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Muriel Moser, declare as follows

1. I am an inventor in the above-identified application.

2. The following experiments evidence that proliferating DCs provide better fusion partners for DC/tumor cell fusions and that the DC/tumor cell fusions formed are capable of providing immunostimulation. First, the phenotype of bone marrow progenitors at different times of culture was characterized (in presence of GM-CSF) and proliferation was monitored. The data is shown in Figures 1-3 attached. Figures 1-2 show evolution of several markers over the 9 day culture period: MHC-II, CD11c, F4/80, GR1 (Ly6G), CD90.2, CD4, CD8, and B220/CD45R. These results show that dendritic cells derived from bone marrow (BMDCs) are CD11c, MHC-II and F4/80 positive and 50% are GR1 positive. These BMDCs do not express B-cell or T-cell

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markers (except CD90.2 at a negligible level). DC specific markers occur early in culture at day 2. 90% of cells are CD11c positive at day 4 and MHC-II positive at day 6.

3. In order to follow cell divisions, progenitor cells were labeled at D0 with CFSE, a cytoplasmic dye which divides equally into daughter cells and therefore decreases proportionally to cell division. The FACS analysis (Figure 3) shows the decrease of CFSE labeling over the course of culture. Cells were also stained for CD11c, MHC-II, F4/80, GR1, B220, CD4 and CD8 molecules. Cell divisions occurred mainly during the first 3 days of culture as shown by the left shift of CFSE staining. The rate of division seems to decrease significantly at day 4, which correlated with the expression of CD11c and F4/80 on almost all cells. At day 3, the high proportion of FL2 negative cells suggested that progenitor cells are dividing and remain poorly differentiated. Thus, cells at day 3 are proliferating. Conversely, at day 4, most cells are CD11c, F4/80, and to a lesser extent, MHC-II and GR1 positive.

4. Fusion experiments were performed using the BMDCs characterized above in paragraphs 2-3. Fusion of early BMDCs (days 3 and 4 of culture) was compared to fully differentiated BMDCs (day 9 of culture). HAT sensitive P815 tumor cells and BMDCs harvested at day 3, 4 or 9 of culture (end of culture) were mixed at 1:1 ratio (10^7 BMDCs and 10^7 P815), washed in serum free DMEM medium at 37°C and spun down. The cell pellet was broken by gentle agitation with a 2 ml serological pipet in presence of 500 µl of PEG 1500 (Boeringher Mannheim). 500 µl of DMEM medium (37°C) were added 1.5 min later. Increasing volumes of DMEM (1, 2, 4 ml) were added every 1.5 min. Cells were then spun down, diluted in PBS BSA (1%)-EDTA (10 mM) and sorted by magnetic microbeads linked to anti-CD11c antibodies, diluted in complete HAT medium and plated. After 24 hours of culture, hybrids were cloned by limiting dilutions in 96 well plates at 0,3 and 1 cell per well in complete HAT medium.

5. ***Fusion yield***

The table shows that higher numbers of hybrid cells were obtained after fusion between P815 and BMDCs at day 3 of culture than with BMDCs at days 4 or 9 of culture, confirming that proliferating DCs are more efficient in DC/tumor cell fusions.

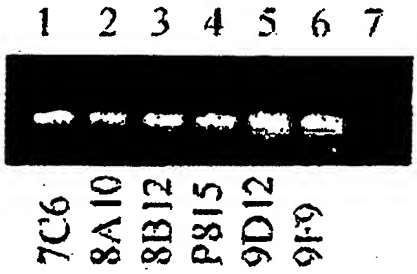
MD

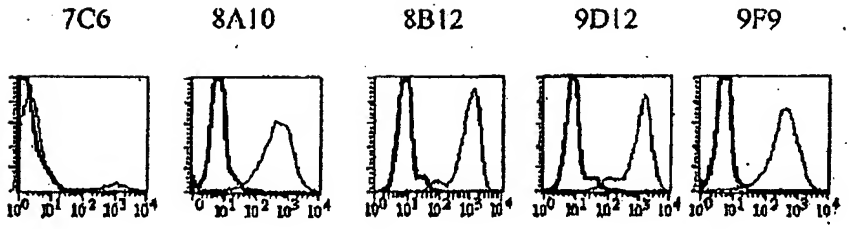
	Number of clones obtained with BMDCs at day 3 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 4 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 9 of culture in 3X96 well plates (1 cell/well)
Exp 1	54	12	-
Exp 2	28	5	1
Exp 3	135	5	-

6. ***Phenotype analysis of hybrid cells***

The fused cells were analyzed to determine that they were true hybrids between DCs (defined as CD11c positive) and P815 tumor cells. CD11c expression was analyzed by flow cytometry and expression of mRNA specific for P815-associated antigen P1A was assessed by RT-PCR. Among 27 clones (exp 3), 26 were CD11c+ and 19 were P1A+.

Example of phenotype analysis for 5 clones





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mRNA expression of P1A is shown for the five clones (lanes 1, 2, 3, 5, 6), P815 tumor cells (lane 4) and 3B4 irrelevant cell, the negative control (lane 7). All clones express tumor antigen P1A. The FACS analysis shows that four clones express CD11c.

7. We conclude that fusion of P815 tumor cells and BMDCs cultured for only 3 days, while the DCs are proliferating, produces more hybrid cells than fusion with BMDCs cultured from 4 or 9 days. Thus, we conclude that it is preferable to use proliferating dendritic cells to make DC/tumor cell fusions because the yield of fused cells is much higher when proliferating DCs are used.

8. Allo-MLRs were performed to assess the immunostimulatory properties of hybrid cells. Hybrid cells (I-Ad) treated or not with LPS (50 μg/ml overnight) were cultured with purified naive T cells (I-Ab). Results from testing of 19 P1A+/CD11c+ hybrid clones:

2 exhibit poor immunostimulatory properties

4 exhibit weak immunostimulatory properties

13 exhibit strong immunostimulatory properties

We conclude that DC/tumor cell fusions may be produced efficiently using proliferating DCs isolated from bone marrow and that these fused cells have strong immunostimulatory properties.

9. We also conducted experiments using different sources for isolation of DCs. While paragraphs 1-8 above describe procedures using DCs from bone marrow, sources such as blood, lymph, lymph nodes and spleen may also be used. We have found that bone marrow, blood and lymph contain a low number of differentiated DCs, but a high number of DC progenitors. Spleen and lymph nodes contain a high number of differentiated DCs and a low number of DC progenitors. As demonstrated in the instant specification, DCs isolated from spleen produced a T-cell/tumor cell hybridoma, not a DC/tumor cell hybrid (see Examples 1-6 and paragraph 0184 of the published application). Further experiments in my laboratory have confirmed that spleen is a poor source for DCs to produce DC/tumor cell hybrids while bone marrow, blood and lymph are the preferred sources.

MM

10. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States codes and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: February 4, 2005 By: Muriel Moser

Muriel Moser

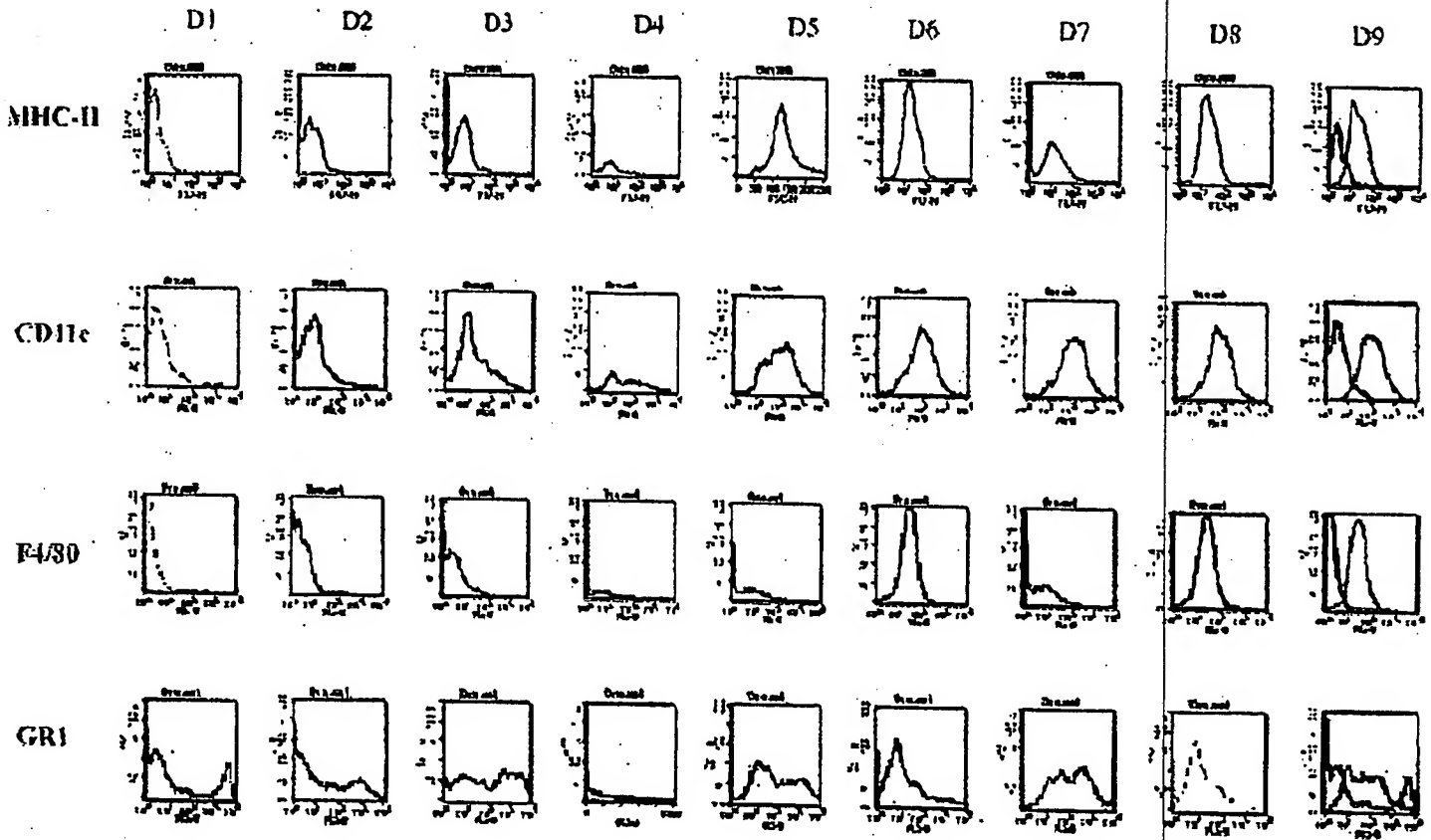

FIGURE 1

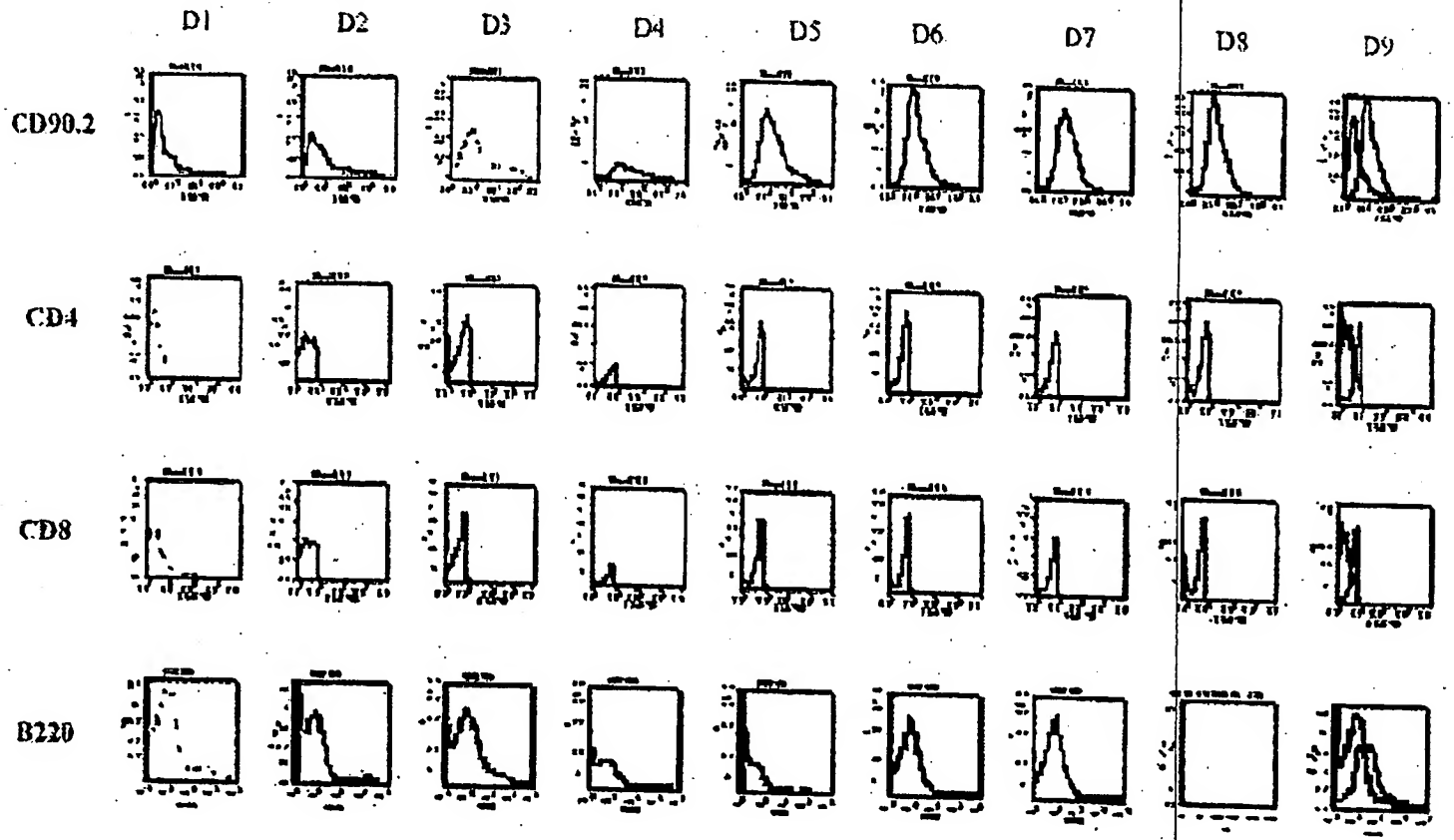

FIGURE 2

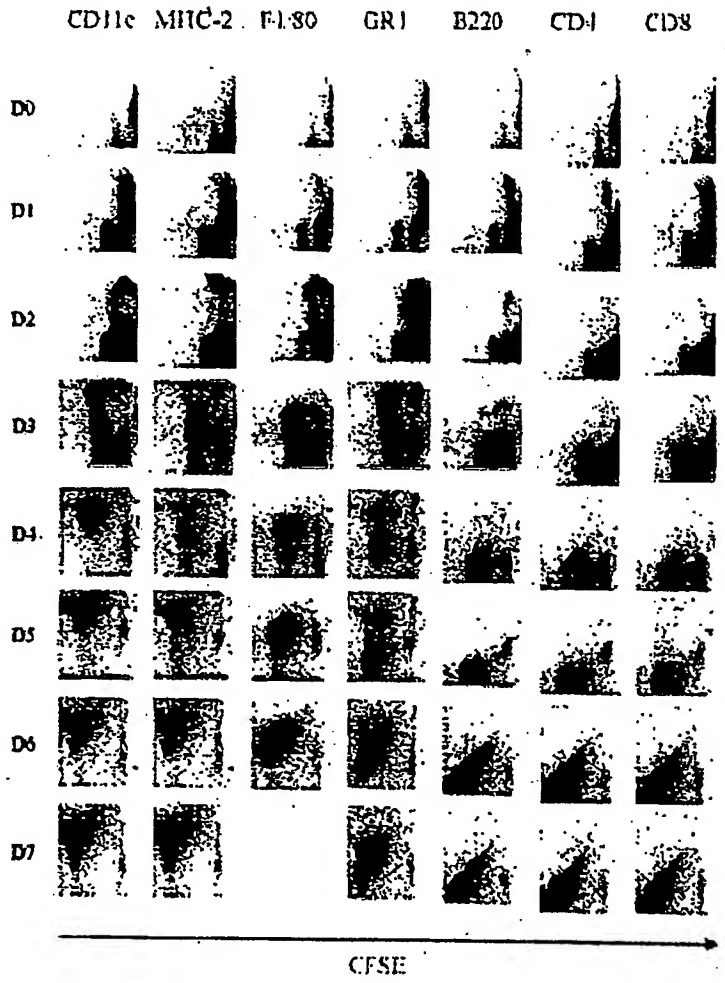

FIGURE 3

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ATTACHMENT F

washed and added to pokeweed or antigen (SKSD; tetanus toxoid) stimulated lymphocyte cultures. The effect of the pre-incubated monocytes was measured by reverse hemolytic plaque assay and ^{3}H thymidine incorporation. Ig synthesis and ^{3}H thimidine incorporation was reduced by 85 % in cultures where monocytes pre-incubated with anti DR sera were added. Monocytes pre-incubated with anti HLA-A sera had essentially no effect. When T cells, isolated by E-rosetting from 24 hour cultures of anti DRw treated monocytes were added to pokeweed or antigen stimulated cultures significant suppression of response was observed. When monocytes «pulsed» with F(ab')2 fragments of allo and hetero DRw sera were added to antigen and pokeweed stimulated cultures, no suppression was observed. The results of these studies indicate that anti-DRw sera can exert an immunoregulatory effect by induction of T suppressor cells.

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Dendritic cell (DC) hybridoma action on T lymphocyte proliferation

J. H. PETERS

Dendritic cells (DC's) may be the primary inducer cells for T lymphocyte growth, stimulated either by antigens or mitogens. Investigation of the detailed DC function is hampered by the rareness of these cells and the absence of tumor lines. For this reason, a number of DC hybridomas has been prepared which retained selected and combined DC properties. CBA mouse DC's were prepared according to a recently developed technique (J. H. PETERS. 1980. Immunobiol. 157: 261). They demonstrated little or no phagocytosis, were adherent to hydrophobic surfaces and weakly positive for unspecific esterase (without prior fixation). They were fused with either BW 5147 lymphosarkoma cells from AKR mice or with P3×63AG8 plasmocytoma cells from BALB/C mice. In order to select for hybridomas approaching the DC phenotype, the cells were kept on hydrophobic surfaces (Petriperm, Heraeus) and non-adherent cells were continuously eliminated. By this regimen 14 different adherent lines could be isolated which in part grew very slowly. Some of them have been successfully cloned. The lines differ markedly in their phenotype, both morphologically and functionally. Three of the tested lines exhibit a strong inductive capacity on T lymphocyte growth, in the absence and/or presence of mitogen. One of them stains positive with anti Ia serum, two are negative. Further data will be given as how far surface markers characteristic of DC's cosegregate with the lymphocyte stimulatory function of DC hybridomas.

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Is interaction of macrophages with other cells mediated by gangliosides?

*M. RIEDL, *O. FÖRSTER, and **H. BERNHEIMER

Gangliosides (G) have recently been implicated in a number of immunological phenomena. E.g., they seem to be receptors for type II interferon (1), they were correlated with murine

ATTACHMENT G

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112. Adherent cell heterogeneity (I): non phagocytic adherent cells in mouse spleen

J. H. PETERS

High macrophage numbers are in vivo elicited into the mouse peritoneal cavity after i.p. injection of thioglycollate medium or other compounds. These macrophages are recruited from the total organism which by this technique becomes widely depleted of macrophages. I have tested how far this macrophage depletion alters the mitogenic response of mouse spleen cells. The experiments revealed that spleen cell cultures of macrophage depleted BALB/c mice show a high mitogenic response on addition of T-cell-specific mitogens, which is frequently higher than the response of normal control spleens. Macrophage-depleted spleens still contain a small fraction of esterase-positive, adherent cells, which are non phagocytic. Mitogenic cooperation with T cells can therefore be attributed to this cell class. This is substantiated by finding of a method to remove this cell from lymphocytes, which then become non-reactive to mitogens (see abstract MÜLLER-HERMES and PETERS). The cooperating cell is loosely adherent, slowly motile, stains positive for nonspecific esterase and tends to spontaneously form rosettes with syngeneic lymphocytes. Thus the criterion of adherence is not sufficient to separate these cells from T cells. They may be identical with the «dendritic cells» described by STEINMAN and COHN: J. Exp. Med. 137 (1973) 1142.

Inst. f. Genetik, Köln[1], Federal Republic of Germany, and Inst. f. Zoophysiology, Uppsala[2], Sweden

113. Adherent cell heterogeneity (II): non-phagocytic, esterase-positive adherent cells in the peripheral human blood

J. H. PETERS, G. OCKLIND, and K. LINDAHL-KIESSLING[2]

Adherent, esterase-positive cells from peripheral human blood are usually regarded as monocytes belonging to the class of macrophages. Monocytes may develop to macrophages upon prolonged culturing in vitro. Both cell types are phagocytic, esterase-positive, and strongly adhere to hydrophobic and hydrophilic surfaces. Here we report about the isolation of a cell type from peripheral human blood which is esterase-positive, slightly adherent, but non phagocytic. Buffy-coat cells are cultivated in RPMI-1640 medium containing 2% fetal-calf serum at 10^7 cells/10 ml in 9 cm siliconized glass petri dishes. After two hours of attachment non-attached cells (lymphocytes, erythrocytes) are removed by repeated gentle washings with culture medium. Adherent cells are further cultured about 15 hrs, after which most of the adherent granulocytes have died and typical monocytes are still attached. However, a subset of previously attached cells has now detached from the surface and thus can easily be separated from monocytes. These cells are esterase-positive, non-phagocytic and they strongly cooperate with T lymphocytes in their mitogenic activation. Furthermore, these cells can be kept in culture for longer times, retaining their esterase and the loosely adherent phenotype. Occasionally the cells grow in size to form mononuclear giant cells. Morphologically and by some of the tested criteria these cells are reminiscent of dendritic cells from mouse spleen.